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54 **Transformed lactic acid bacteria.**

57 An inoculum for silage and a probiotic which include lactic acid bacteria transformed with at least one exogenous gene or DNA fragment thereof coding for an enzyme which breaks down an oligosaccharide and/or a polysaccharide into a monosaccharide, disaccharide or other fermentable carbohydrate. Also provided are methods for transforming the lactic acid bacteria by electroporation and by the use of new plasmids, vectors and other DNA sequences. A new amylase is also provided.

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Description

TRANSFORMED LACTIC ACID BACTERIA

Background of the Invention

This invention relates to bacterial microorganisms, particularly Streptococcus, Lactobacillus, Enterococcus, Lactococcus, Pediococcus and Leuconostoc species, which carry out lactic acid fermentation. This invention particularly relates to the use of such lactic acid bacteria as inocula in silage and as probiotics. This invention more particularly relates to the use in silage and as probiotics of lactic acid bacteria which have been transformed with foreign genes or DNA fragments which code for enzymes that degrade oligosaccharides and/or polysaccharides into monosaccharides, and disaccharides and other carbohydrates fermentable into lactic acid.

Lactic acid bacteria are a non-taxonomic group of Gram-positive non-sporeforming bacteria which ferment simple water soluble carbohydrates ("WSC") to produce lactic acid as the predominant end product. Such bacteria are widely used in the feed, food and dairy industries. Traditionally, they have mainly included species from the genera Lactobacillus, Streptococcus, Leuconostoc and Pediococcus. The taxonomy of these microorganisms has been described in "Bergey's Manual of Systematic Bacteriology" (Sneath et al., 1986). However, the taxonomy of Streptococcus has recently been revised extensively (Schleifer and Kilpper-Bälz, 1987). On the basis of many biochemical and serological studies, the genus Streptococcus has been divided into three new genera: 1) Streptococcus sensu stricto that contains the majority of known streptococcus species, many of which can be pathogenic; 2) Enterococcus that contains the enteric streptococci; and 3) Lactococcus that contains all lactic streptococci. Of these three genera, it is the Lactococcus and Enterococcus which are extensively used in the feed, food and dairy industries.

Silage

Silage is a process of controlled anaerobic fermentation of a crop (e.g., grass, cereals or legumes) in order to preserve it for use as a feed for animals (McDonald, 1981). Proper ensilage depends upon the presence of suitable lactic acid bacteria in order to produce enough lactic acid to cause a rapid and significant pH decline in the fermentation medium. The pH decline stops the growth of undesirable microorganisms such as Clostridia (Woolford & Sawczyk, 1984) in the silage and tends to inhibit post-harvest changes in the silage, particularly in its protein fraction (Woolford, 1984).

The lactic acid bacteria commonly used in ensilage can be divided into two major categories: 1) the homofermentative lactic acid bacteria which ferment predominantly hexoses to lactic acid; and 2) the heterofermentative lactic acid bacteria which ferment hexoses to lactic acid and other products, such as ethanol and acetic acid (Beck, 1978). Both categories of bacteria can be further subdivided into rod and coccus categories (Beck, 1978). The most important homofermentative rod bacteria are: Lactobacillus casei, Lactobacillus coryniformis, subsp. coryniformis, Lactobacillus curvatus, Lactobacillus salvarius, Lactobacillus acidophilus and Lactobacillus plantarum. The most important homofermentative coccus bacteria are: Pediococcus acidilactici, Pediococcus cerevisiae, Pediococcus pentosaceus, Enterococcus faecalis, Lactococcus lactis and Enterococcus faecium. The most important heterofermentative rod bacteria are: Lactobacillus brevis, Lactobacillus buchneri, Lactobacillus fermentum and Lactobacillus viridescens. The most important heterofermentative coccus bacteria are: Leuconostoc cremoris, Leuconostoc citrovorum, Leuconostoc dextranicum and Leuconostoc mesenteroides.

Lactic acid bacteria in a silo normally come from the ensiled vegetation itself or from natural inoculation by farm equipment (McDonald, 1981). To ensure a rapid and reproducible pH decline in silage, cultured lactic acid bacteria, such as one or more of the aforementioned Lactobacillus, Enterococcus, Lactococcus, Pediococcus and/or Leuconostoc species, are often added to a crop prior to ensilage (Seale, 1986).

The majority of lactic acid bacteria can ferment only simple water soluble carbohydrates, such as the monosaccharides, disaccharides and trisaccharides (e.g., glucose, arabinose, maltose, cellobiose, fructose, galactose, lactose, mannose, melizitose, melibiose, raffinose, rhamnose, ribose, sucrose, trehalose and xylose) and related compounds such as mannitol, sorbitol, galacturonate and gluconate, which are part of the WSC of the crop.

However, the WSC content of a crop is variable and depends upon the species of crop, its stage of growth, and the weather and fertilizer to which it has been exposed (Woolford, 1984). In order to increase the WSC content and composition of silage, carbohydrates fermentable to lactic acid by lactic acid bacteria (the "fermentable carbohydrates") are often added to a crop (McDonald, 1981), either in the form of: WSC-rich vegetation such as maize, barley, oat, wheat or milo; other WSC-rich material such as molasses or whey; or directly in the form of more or less pure fermentable monosaccharides, disaccharides and trisaccharides as mentioned above. These fermentable carbohydrates can be added to the crop either alone or in combination.

Besides adding fermentable carbohydrates to a crop to increase its WSC content, silage has been improved by adding enzymes to the crop to break down polysaccharides and oligosaccharides, whether naturally present in the crop or added to the crop prior to ensilage, which could not otherwise be fermented by the lactic

acid bacteria. In this regard, exogenous enzymes such as amylases, pectinases, cellulases and/or hemicellulases, which can degrade polysaccharides (e.g., starch and cellulose) and/or oligosaccharides into monosaccharides, disaccharides, trisaccharides and/or other fermentable carbohydrates, have been added to crops prior to ensilage. Sometimes, non-fermentable carbohydrates, such as starch, have also been added to a crop, together with a suitable enzyme, such as an amylase, to break down the starch.

The addition of lactic acid bacteria inocula, enzymes and/or sources of fermentable carbohydrates, as described above, has been found to provide better control of the ensiling process, with rapid fermentation to produce lactic acid, with minimal losses of silage during storage and with maximal feed quality of the stored product (Bolton, 1985).

The addition of so-called "cellulases", together with lactic acid bacteria, has been particularly useful (Seale, 1987). Such cellulase additives have been important in breaking down rigid plant cell walls, which consist of complexes of cellulose, hemicellulose, pectin and lignin, and derivatives thereof, and liberating from the cell walls fermentable carbohydrates that would otherwise not be part of the WSC content of the crop. In addition, such cellulase additives are likely to have locally degraded, and thereby perforated, the cell walls so that intracellular WSC of the plant cells has become available for the lactic acid bacteria.

However, these cellulase additives have contained more than just cellulases. Cellulases have classically been divided into three major classes: 1) the endoglucanases which break the beta-1,4-linkage in the amorphous region of cellulose; 2) the exoglucanases or cellobiohydrolases which liberate cellobiose to glucose units from the ends of the glucose-chains; and 3) the beta-glucosidases which degrade cellobiose to glucose. Genes coding for these different cellulases are known (Knowles et al, 1987). It is now believed that many of these cellulase additives have contained, besides cellulases, proteins with no known enzymatic activity (i.e., non-enzymatic proteins) which have promoted the action of the cellulases in the additives in degrading cellulose in the silage. In this regard, it has long been known that although cellulose is a chemically simple compound, it is a physically complex beta-1,4-linked glucose polysaccharide which consists of crystalline cellulose regions linked by amorphous cellulose regions (Coughlan, 1985). For this reason, natural organisms which degrade cellulose have generally been found to, possess a multiprotein system, including different enzymatic and non-enzymatic proteins that act synergistically and, at least in some bacteria, are physically aggregated in so-called cellulosomes. Recent studies also have demonstrated that, at least in some cases, only two cellulases are required to obtain a substantial breakdown of cellulose (Wong et al 1988) and the use of only one cellulase, plus a non-enzymatic protein which promotes the degradation of cellulose by the cellulase, has been found to break down substantially crystalline cellulose (Wu et al, 1988). Thus as used herein, the term "enzyme that degrades a polysaccharide and/or an oligosaccharide" means an enzyme in the classic sense (e.g., a cellulase) which degrades a polysaccharide and/or an oligosaccharide, as well as the non-enzymatic proteins which promote the degradation of polysaccharides and/or the oligosaccharide by the enzyme.

Probiotics

Lactic acid bacteria, particularly Enterococcus and Lactobacillus, also serve a key role in establishing and maintaining optimal intestinal flora in animals. The intestinal tracts of animals constitute a very specific ecological system colonized by a mixed population of microorganisms whose balance plays an essential role in the animals' health and growth (Raibaud and Ducluzeau, 1984). The spectrum of species and genera of microorganisms in such systems varies greatly from one animal species to another. Potentially harmful germs, such as Escherichia and Salmonella, cohabit with beneficial bacteria such as Enterococcus and Lactobacillus (Vrignaud, 1982). Their equilibrium is controlled by both external factors (mainly food quality and hygiene) and internal parameters such as stress. Severe disruption of this equilibrium can lead to diarrhea which is a significant cause of death among young farm animals such as piglets and calves (Vrignaud, 1982).

Probiotics are food additives which help to establish and maintain an optimal intestinal flora in man and other animals. Lactic acid bacteria are considered to be preferred bacterial probiotics (Fernandes et al, 1987). The beneficial effects of lactic acid bacteria is primarily due to their positive role in natural intestinal flora. By producing lactic acid, such bacteria lower the pH in the intestine, thereby setting up unfavorable conditions for the proliferation of most gram-negative pathogens. In addition, many species of lactic acid bacteria secrete compounds, known as bacteriocins (e.g., nisin produced by many strains of L. lactis), which exhibit strong antibiotic properties. Lactic acid bacteria also produce vitamins (mainly groups B and K) and lower redox potential (Klaenhammer, 1982; Aumaitre, 1988). The use of lactic acid-bacteria probiotics as human food and animal feed supplements has become popular in recent years (Wolter and Henry, 1982; Aumaitre 1988).

It has been demonstrated that the addition of 3-4% of dried lactic acid bacteria to the powdered aliments, fed to piglets and calves, reduces their incidence of diarrhea significantly, lowers their consumption index, and increases their growth (Vrignaud, 1982). It has also been demonstrated that the growth performance of farm animals, such as piglets, calves and chickens, can be significantly enhanced by supplementing their food with enzyme preparations (Lyons, 1987; Chesson, 1987). Enzymes, such as α -amylase, β -glucanase, xylanase and pectinase, have been found to be most useful in this respect (Marquardt et al, 1987). The addition of amylases to the feed of young calves has also been found to facilitate the transition from weaning to solid food (Gilliland, 1988). Not only do such enzymes increase the nutritional value of the diet by improving digestibility (Broz,

1987), but they also decrease the antinutritional value of some feedstuffs (such as rye) by degrading viscous polysaccharide components (e.g., pentosanes) known to interfere with the proper functioning of the digestive tract in chickens (Fengler and Marquardt, 1986).

5 Summary of the Invention

In accordance with this invention, an inoculum for silage is provided, which comprises lactic acid bacteria transformed with at least one exogenous gene or DNA fragment of the gene (the "exogenous DNA") coding for an enzyme which breaks down an oligosaccharide and/or a polysaccharide into a fermentable carbohydrate. By the use of this inocula, an ensilage method is provided in which it is not necessary to add
10 exogenous enzymes to the crop and it may not even be necessary to add fermentable carbohydrates to the silage while carrying out the ensilage process in an otherwise conventional manner. In this regard, the transformed lactic acid bacteria of this invention can breakdown polysaccharides and oligosaccharides in the crop itself to provide a complete source of the fermentable carbohydrates that the lactic acid bacteria needs for producing lactic acid.

15 Also in accordance with this invention, a probiotic is provided which comprises lactic acid bacteria transformed with at least one of the exogenous DNAs; provided that when the transformed lactic acid bacteria is a Streptococcus, it is transformed with at least two of the exogenous DNAs which code for different enzymes that can degrade a polysaccharide and/or an oligosaccharide into a fermentable carbohydrate. This probiotic can be used in an otherwise conventional manner in a method for establishing and maintaining
20 optimal intestinal flora in animals, including humans.

Further in accordance with this invention are provided lactic acid bacteria transformed with at least one, preferably at least two, of the exogenous DNAs; provided that when the transformed lactic acid bacteria are Streptococcus, they are transformed with at least two of the exogenous DNAs which code for different enzymes that can degrade a polysaccharide and/or oligosaccharide into a fermentable carbohydrate.

25 Still further in accordance with this invention, an electroporation method is provided for transforming lactic acid bacteria, including the use of new plasmids, vectors and DNA sequences.

Yet further in accordance with this invention, a new amylase is provided, having an amino acid sequence as shown in Fig. 2. In addition, a DNA sequence, encoding the new amylase, is provided as also shown in Fig. 2, particularly from nucleotide 184 to nucleotide 2131.

30 Description of the Invention

In transforming lactic acid bacteria for use as an inoculum for silage or as a probiotic, particularly for silage, any lactic acid bacteria can be used in accordance with this invention. However, when the lactic acid bacteria is a Streptococcus that is to be used as a probiotic, the Streptococcus is transformed with at least two of the
35 exogenous DNAs, and when the lactic acid bacteria is a Lactobacillus transformed with an exogenous DNA coding for an amylase, the Lactobacillus is preferably transformed also with at least one other exogenous DNA coding for a different enzyme, preferably a cellulase. The preferred species of lactic acid bacteria for silage are from the genera Lactobacillus, Enterococcus, Lactococcus, Pediococcus and Leuconostoc, all of which have been used previously in silage. The particularly preferred species for silage are from the genera Enterococcus,
40 Lactobacillus and Pediococcus, such as the aforementioned species of such genera. The preferred species for probiotics are from the genera Enterococcus and Lactobacillus.

The lactic acid bacteria can be transformed with any foreign gene or DNA fragment thereof which codes for the expression and secretion of an enzyme that can degrade a polysaccharide and/or an oligosaccharide to a fermentable carbohydrate. In this regard, the foreign gene or DNA fragment can code for: an enzyme that can
45 degrade starch, i.e., an amylase such as an α -amylase, β -amylase, glucoamylase, isoamylase or pullulanase; an enzyme that can degrade cellulose, i.e., a cellulase such as an endoglucanase, exoglucanase or beta-glucosidase; an enzyme that can degrade pectin, i.e., a pectinase such as a pectic lyase, pectic glycosidase or a pectic methyl esterase; or an enzyme that can degrade hemicellulose, i.e., a hemicellulase such as a xylanase, mannanase, arabinanase, inulinase, levanase or fructanase.

50 For many uses as an inoculum for silage or as a probiotic, the lactic acid bacteria is preferably transformed with 2 or more (e.g., up to 5) of these exogenous DNAs coding for different enzymes. Each of the different enzymes, encoded by the plurality of exogenous DNAs, can degrade a polysaccharide or oligosaccharide into a fermentable carbohydrate. Alternatively, one or more of the different enzymes can degrade a polysaccharide or oligosaccharide into a simpler saccharide, and one or more of the other different enzymes can degrade the
55 simpler saccharide into a fermentable carbohydrate, so long as the overall action of the different enzymes is to degrade a polysaccharide or oligosaccharide into a fermentable carbohydrate. In any event, a plurality of DNAs will preferably be selected to provide the transformed lactic acid bacteria with a suitable combination of enzymes for degrading the intended substrate (e.g., the crop or the animal feed) to obtain an increase in fermentable carbohydrates. Lactic acid bacteria for silage are preferably transformed with exogenous DNAs
60 coding for at least one cellulase, especially two or more cellulases (e.g., an endoglucanase and an exoglucanase). Lactic acid bacteria for a probiotic are preferably transformed with at least one exogenous DNA coding for an amylase.

For any lactic acid bacteria, as well as any other microorganism, transformed with two or more exogenous DNAs, one of which codes for an enzyme, particularly an amylase, in accordance with this invention, the
65 enzyme encoded by the exogenous DNA can serve as a marker for transformed microorganisms. Thereby, a

method for detecting transformed microorganisms of this invention is provided, which involves detecting the enzyme produced by the transformed microorganisms.

Suitable exogenous DNAs of this invention, coding for enzymes that degrade cellulose, can be obtained from prokaryotic or eukaryotic microorganisms such as:

the eukaryotic fungi: Trichoderma, Aspergillus, Agaricus, Botryodiplodia, Chaetomium, Eupenicillium, Fusarium, Humicola, Macrohomina, Myrothecium, Myceliophthora, Pellicularia, Penicillium, Pestalotiopsis, Polyporus, Poria, Sporotrichum, Talaromyces, Thermoascus, Thielavia, Tranetes, Trichosporum, Mucor, Torula, Neurospora, Paecilomyces, Lipomyces, Schwanniomyces, Torulopsis, Endomycopsis, Rhizoctonia, Rhizopus, Coniothyrium, Corticium, Verticillium and Monilia;

the prokaryotic bacteria: Acetivibrio, Bacteroides, Bacillus, Cellulomonas, Cellvibrio, Clostridium, Microbispura, Plectridium, Erwinia, Pseudomonas, Selenomonas, Butyrivibrio, Eubacterium, Aerobacter, Micrococcus, Acinetobacter, Cytophaga, Escherichia, Myxobacter, Xanthomonas, Alternaria and Ruminococcus;

the prokaryotic actinomycetes: Streptomyces, Thermoactinomyces, Thermomonospora and Caldocellum; and

the eukaryotic yeasts: Kluyveromyces, Candida, Saccharomyces and Schizosaccharomyces.

Suitable exogenous DNAs, coding for enzymes that degrade starch, can be obtained from a large variety of prokaryotes such as Bacillus, Streptomyces, Clostridium, Klebsiella, Streptococcus and lactic acid bacteria such as Lactobacillus amylophilus, Lactobacillus amylovorus, Lactobacillus cellobiosus and Enterococcus, from fungi such as Candida japonica, Endomycopsis, Lipomyces, Schwanniomyces, Aspergillus niger and Rhizopus and from most higher plants and animals.

Suitable exogenous DNAs, coding for enzymes that degrade pectins, can be obtained from prokaryotes such as Pseudomonas marginalis, Bacillus, Clostridium, Erwinia, Cytophaga, Xanthomonas, Aeromonas, Flavobacterium and Streptomyces, from various plant pathogenic fungi and saprophytic fungi, and from most higher plants.

Suitable exogenous DNAs, coding for enzymes that degrade hemicellulose, can be obtained from bacteria such as Streptomyces, Bacillus, Sporocytophaga myxococcoides, Clostridium, Butyrivibrio fibrisolvens, Ruminococcus albus and R. flavefaciens, from protozoa such as Epidinium ecaudatum, Entodinium and Eremoplastron bovis, and from a wide variety of plant pathogenic and saprophytic fungi.

Needless to say, other microorganisms can provide suitable exogenous DNAs for transforming lactic acid bacteria in accordance with this invention. Furthermore, one can transform lactic acid bacteria: with an exogenous DNA produced from a messenger RNA corresponding to a naturally produced gene or DNA fragment thereof; or when the sequence of a desired exogenous DNA has been established, with a synthetically produced exogenous DNA. Of course, exogenous DNA obtained by a combination of such methods can also be used. Thus, terms such as "gene", "foreign DNA fragment thereof" and "exogenous DNA", as used herein, are not limited to naturally occurring genes and DNA fragments thereof which encode natural enzymes that degrade polysaccharides and/or oligosaccharides. These terms also cover other nucleic acids, such as cDNAs, chimeric genes and synthetic nucleic acids, encoding the same or similar enzymes.

Further, it goes without saying that each exogenous DNA used for the transformation of lactic acid bacteria must cause the bacteria to express and secrete the enzyme encoded by the exogenous DNA. This means that the exogenous DNA has to contain regulatory sequences such as a suitable promoter, secretion signal, translation initiation signal and termination signal operable in the lactic acid bacteria. These regulatory sequences may be homologous to the enzyme encoding sequences, i.e., naturally associated with such a coding sequence. However, this invention also extends to so called chimeric exogenous DNAs, i.e., DNAs in which one or more regulatory sequences are not normally associated with the enzyme encoding sequence. In this regard, the "PRLB" promoter of Fig. 7a is a preferred heterologous promoter for controlling an exogenous DNA of this invention in lactic acid bacteria.

For transforming lactic acid bacteria, each exogenous DNA can be provided in an episomal vector, e.g., a plasmid. Well known process techniques can then be used to carry out such a transformation with the vector to achieve high levels of expression of the exogenous DNA of this invention. Cultivating cultures of such transformed lactic acid bacteria also can be carried out in a conventional manner. Of course, segregation of vectors during cell division occurs at random, and cells will appear which do not carry a vector with one or more of the exogenous DNAs of this invention cloned on them. As a result, cells without such a vector could eventually predominate in cultures of the bacteria in which there is no selective pressure for carrying cells with the vector. Nevertheless, a population of bacteria in which some bacteria carry the vector will be at an advantage under silage conditions. Indeed, since the growth of bacteria is limited by the supply of WSC in silage, bacteria carrying the vector, enabling them to degrade plant oligosaccharides and/or polysaccharides, will be assured of an increased supply of WSC. Thus, vectors carrying exogenous DNAs for degrading plant polysaccharides and/or oligosaccharides may be maintained in a silage environment, even though outside the silage environment, such vectors would probably be lost at a high rate.

However, it is preferred that, after transformation of the lactic acid bacteria, each exogenous DNA of this invention be inserted in the bacterial chromosome. This provides more stable transformants and can be

suitably accomplished as follows Each exogenous DNA can be first inserted in a bacterial transposon. The transposon also carries a selectable or screenable marker gene and is cloned into a plasmid which cannot replicate in the lactic acid bacteria or whose replication can be inhibited in the bacteria. The plasmid is then introduced in the lactic acid bacteria, and the transformants are screened for the presence of the marker gene so that only bacteria, in which the transposon has moved to the chromosome, will be selected. Alternatively, a plasmid can be constructed which itself contains a marker gene and a part of the bacterial chromosome and which also cannot replicate in the lactic acid bacteria. After introduction of the exogenous DNA in the plasmid, transformation of the lactic acid bacteria and screening for the marker gene, only those bacteria are selected in which the plasmid has been inserted in the bacterial chromosome through a single recombination event between the homologous DNA regions of the plasmid and the bacterial chromosome. If the exogenous DNA and the marker gene are introduced within the bacterial chromosomal region of the vector, the plasmid can also be constructed so that, after transformation of the lactic acid bacteria, the exogenous DNA is inserted in the bacterial chromosome through a double homologous recombination event. Of course, the choice of a suitable region of the bacterial chromosome, which is to be cloned in the plasmid, must be such that the later integration of the exogenous DNA in the chromosome does not hinder the bacteria in their growth or other essential activities.

The transformed lactic acid bacteria of this invention can be used in silage in a conventional manner and in conventional amounts. For example, about 10^5 to 10^6 viable bacteria per gram of silage will generally be a minimum, and about 10^7 bacteria per gram of silage will generally be a maximum. Preferably, the expression of the foreign genes incorporated in the lactic acid bacteria will: 1) increase the amount of fermentable carbohydrates to about 10 to 35 grams per kilogram of silage, preferably to an amount of 15 to 25 grams per kilogram of silage; and 2) lower the pH in the silage to a value of about 3 to 5, preferably to a value of 3.5 to 4.5, within a period of about 24 to 120 hours, preferably 24 to 72 hours.

For probiotics of this invention, the lactic acid bacteria is preferably transformed with an exogenous DNA fragment that encodes an amylase. This is because starch is included in most formulations of food for humans and industrial feed for farm animals and constitutes the major part of some natural aliments (e.g., grain for chicken). Thus, an amylase, produced by a lactic acid bacteria probiotic in the intestinal tract of the human or animal, will hydrolyse residual starch remaining undigested after the action of amylases from the human's or animal's saliva, thereby increasing the energy value of most food and feed formulations and/or allowing the use of less expensive formulations.

Also in probiotics, the lactic acid bacteria preferably is derived from a bacteria found in the natural intestinal flora of animals, such as *Enterococcus* and *Lactobacillus* species (Vrignaud, 1982). Such bacteria can be transformed in a conventional manner with an exogenous DNA which directs the expression and secretion of an enzyme, such as one of the aforementioned enzymes for degrading oligosaccharides and/or polysaccharides into fermentable carbohydrates.

The transformed lactic acid bacteria of this invention can be used as a probiotic in a conventional manner and in conventional amounts. For example, about 10^6 cells per liter (or kg) of food stuff will generally be a minimum, and about 10^{10} to 10^{12} cells per liter of food stuff will generally be a maximum.

The invention further relates to the plasmid designated hereinafter as "pLH1" or "pLAB1000", the nucleotide sequence of which appears in Fig. 3a, and to cloning vectors for lactic acid bacteria which are derived from pHL1 and contain at least the regions of this plasmid necessary for replication in lactic acid bacteria. While this plasmid and its derivatives find preferred use for the production of lactic acid bacteria which produce enzymes which degrade polysaccharides and/or oligosaccharides into fermentable carbohydrates, they can also be used as vectors for the production of transformed lactic acid bacteria which, in cell culture media, produce other proteins. In this regard, the invention concerns plasmids derived from pLH1 in which other nucleotide sequences, preferably coding for determined polypeptides that are to be produced in lactic acid bacteria, are cloned.

The invention also extends to modified plasmids derived from pLH1 and containing a nucleotide sequence foreign to that plasmid and coding for a determined polypeptide inserted either within an exogenous DNA of the present invention, preferably an amylase, or in another part of the plasmid under the control of its own regulation sequences. In the latter case, a sequence encoding amylase can then be used as a screenable transformation marker. In other words, bacteria transformed with the so-modified plasmid and expressing the foreign nucleotide sequence can be detected by their amylase producing capability.

The invention also relates to plasmids derived from pLH1 which comprise a promoter operable in lactic acid bacteria, such as the PRLB promoter of *Lactobacillus hilgardii*, the DNA sequence of which is shown in Fig. 7a (Platteeuw, 1986). DNA fragments, which are normally not expressible in lactic acid bacteria due to the absence of a suitable promoter, can be easily cloned in these pLH1-derived expression vectors to achieve expression of the DNA fragments in these bacteria.

The invention still further relates to a process for transforming lactic acid bacteria with one or more exogenous DNAs by electroporation. Preferably, electroporation is carried out with rapidly growing, exponential phase, bacterial cells, particularly as measured at an O.D.₆₀₀ equal to 0.2 to 0.8, preferably 0.4 to 0.6. It is also preferred that the cells be washed, immediately before electroporation, with water or conventional buffers of low ionic strength. It is further preferred that the electric field be applied to a concentrated suspension of the cells of 10^{10} - 10^{11} Colony Forming Units ("CFU")/ml in a low ionic strength buffer. This process avoids the need for forming protoplasts of the lactic acid bacteria before transformation.

The processes for transforming lactic acid bacteria (e.g., Lactobacillus plantarum and Enterococcus faecalis) with one or more exogenous DNAs by inserting such exogenous DNAs in the bacterial chromosomes by means of transposon vectors or by means of homologous recombination will be further described in the Examples which follow and which illustrate the invention. The figures, referred to in the Examples, are as follows:

- Figure 1 Restriction map of the Bacillus thuringiensis amylase gene. 5
- Figure 2 DNA sequence of the B. thuringiensis amylase gene and the amino acid sequence of the amylase encoded by the DNA sequence.
- Figure 3 a. Nucleotide sequence of the pLH1 or pLAB1000 plasmid. 10
- b. Physical map of the pLAB1000 plasmid (with different numbering from Fig. 3a).
- c.
- A. homology of ORF 1 with other site-specific recombinases.
- B. homology of RS_A with target size of other site-specific recombinases.
- d.
- A. homology of ORF 2 with replication proteins of other organisms. 15
- B. homology of nicking site with those of other organisms.
- Figure 4 Construction of pGI4010, pGI4011 and pERM 3.2 derived from pLAB1000.
- Figure 5 Construction of pRAM11 and pRAM12.
- Figure 6 Construction of pST11 and pST12.
- Figure 7 a. Sequence of PRLB promoter. 20
- b. Construction of pPGV1LH.
- c. Construction of pGC11 and pGC12.
- Figure 8 Construction of pH421.
- Figure 9 Construction of pSATE253 and pSATE256.
- Figure 10 Construction of pTnAMYL. 25
- Figure 11 Construction of pV α .
- Figure 12 Construction of pVA α .
- Figure 13 Construction of pTV α .
- Figure 14 Construction of pG18 α .
- Figure 15 Construction of PG18 ϕ α . Abbreviations used in the figures and throughout the examples are: 30
- Em, Em^R, ERM, ERY, Erythro : erythromycin resistance gene
 - Amp, Amp^R, BLA : Ampicillin resistance gene
 - Cm, Cm^R, Cam, Cml : Chloramphenicol resistance gene
 - Km^R, Kan : kanamycin resistance gene
 - Tc, tet^R, Tet : tetracyclin resistance gene 35
 - ① : polylinker
 - ori : replication origin (ori⁻ : gram negative bacteria; ori⁺ : gram positive bacteria)
 - amy, α : α -amylase gene from Bacillus stearothermophilus
 - celA : endoglucanase gene from Clostridium thermocellum
 - lacpo : lac promoter 40
 - cceg : DNA from Clostridium cellulolyticum containing an endoglucanase gene
 - Lp80 DNA : chromosomal DNA from Lactobacillus plantarum 80
 - PL : P_L promoter from phage lambda
 - LB : pLH1 sequence
- Unless otherwise stated in the Examples, all procedures for making and manipulating recombinant DNA were carried out by the standardized procedures described in Maniatis et al, Molecular Cloning - A Laboratory Manual, Cold Spring Harbor Laboratory (1982). Unless otherwise stated, the Escherichia coli used for transformations was E. coli strain MC 1061 (Casadaban and Cohen, 1980).
- The following Lactobacilli, used in the Examples, have been deposited in the Deutsche Sammlung Von Mikroorganismen (the "DSM"), Göttingen, Federal Republic of Germany, under the provisions of the Budapest Treaty: 50

Microorganism	DSM Accession No.	Date
<u>L. plantarum</u> 80	4234	27 August 1987
<u>L. plantarum</u> 50	4429	27 August 1987
<u>L. hilgardii</u> 67	4233	27 August 1987
LpGA39	4775	29 August 1987

The other microorganisms in the Examples are also readily available or can readily be reproduced from readily available microorganisms and genetic material without performing any inventive work.

Example 1: Cloning an α -amylase gene from *Bacillus stearothermophilus*

The α -amylase gene from *B. stearothermophilus* strain CU21 has been characterized and its DNA sequence determined (Aiba et al, 1983; Nakajima et al, 1985). Total DNA was prepared from *B. stearothermophilus* CU21 as described by Aiba et al. (1983), digested with PvuII and HindIII, and size fractionated on an agarose gel. The 3 kilobase ("kb") pair size fraction was recovered, and DNA was purified from this size fraction and ligated to plasmid pUC9 (Messing, 1980) which was digested with HindIII and SmaI. The ligation mixture was transformed into *Escherichia coli* strain JM83 (Yanish-Perron et al, 1985). Transformed cells were plated on LB agar with 100 mg/l ampicillin and 1% (by weight) soluble starch (Difco Laboratories, Detroit, Michigan, U.S.A.) and were incubated overnight at 37°C. The agar plates were then stained with iodine vapor. A halo of non-staining agar around certain colonies was indicative of amylase activity. These colonies were picked and colony purified, and plasmid DNA was prepared from them.

The DNA sequence of the insert in one of these plasmids named "pSTUC" (Fig. 5), was determined by the Maxam and Gilbert chemical degradation method (Maxam and Gilbert, 1980) and was found to be identical to the DNA sequence published previously for the α -amylase gene from this strain (Nakajima et al, 1985).

Example 2: Cloning an amylase gene from *Bacillus thuringiensis* (Figs. 1 and 2)

Total DNA was prepared (Aiba et al, 1983) from *B. thuringiensis* strain H1.1, serotype H1, var. *thuringiensis*, obtained from Dr. Huguet de Barjac, Institut Pasteur, Paris. It was digested with restriction enzyme HindIII and ligated to bacteriophage lambda cloning vector 590 (Murray et al, 1977) cleaved with HindIII. The ligation was packaged, infected on *E. coli* and plated on LB agar supplemented with 1% soluble starch. Phage containing the *B. thuringiensis* amylase gene were identified by the halo they form after staining in iodine vapor. The existence of these halos proved the expression of the amylase gene in *E. coli*.

From one such phage, having a 5.6 kb pair insert, a restriction map was determined, and the amylase gene was localized more precisely by subcloning restriction fragments of its insert in plasmid pBR322 (Bolivar and Backmann, 1979) and testing amylase expression in *E. coli* HB101 (Bolivar and Backmann, 1979). A 2.55 kilobase pair PvuII-HindIII restriction fragment of the 5.6 kb pair insert, cloned in pBR322, exhibited strong amylase activity on agar plates supplemented with 1% soluble starch. This pBR322 clone was named "pJAM23". The DNA sequence of this fragment insert in pJAM23 was shown by sodium dodecyl sulfate ("SDS") polyacrylamide electrophoresis of *E. coli* cell lysates to encode a 76,000 dalton protein.

A restriction map of this fragment, including the amylase gene, is given in Fig. 1. The DNA sequence of most of the fragment insert was defined by the Maxam and Gilbert chemical degradation method and is given in Fig. 2. There is only one big open reading frame ("ORF"), oriented from the HindIII site towards the PvuII site. This encodes a 76,059 dalton polypeptide consisting of 648 amino acids. No homology was found to other α - or β - amylases such as those described in Nakajima et al (1986), Nishizawa et al (1987) and Kawazu et al (1987).

Downstream of the *B. thuringiensis* amylase gene is a sequence that is highly homologous on the amino acid level to the *E. coli* ADP glucose transferase gene (Baecker et al, 1983).

Example 3: Isolating and characterizing a *Lactobacillus* plasmid (Fig. 3)

Lactobacillus hilgardii 67 was isolated from grass silage by Radar N.V. (Deinze, Belgium). The strain was grown in static culture at 37°C in MRS medium (made by Oxoid Ltd., Basingstoke, Hampshire, England) supplemented with 20 mM D-L-threonine. Cells were pelleted from a late log phase culture, resuspended in 10 mM Tris-HCl pH 7.0, pelleted, resuspended in the same buffer and pelleted again. 0.2 gram of cells was resuspended in 2 ml of 20 mM Tris-HCl pH 7.0, 2ml of a 24% solution of polyethylene glycol in water were added, and 34 enzyme units of mutanolysine (Sigma Chemical Company, St. Louis, MO, USA) in water were added. The solution was incubated at 62°C for 1 hour. 0.4 mg of Proteinase K (Sigma) was added, and the cells were incubated 60 minutes at 37°C to form protoplasts.

The protoplasts were collected from the mixture by centrifugation and resuspended in 5 ml of 20 mM Tris-HCl and 10 mM ethylene diamine tetraacetate ("EDTA") at pH 8.0. SDS was added to 3% and mixed with the protoplasts which were then left at room temperature (25°C) for 30 minutes. The resulting cell lysate was centrifuged to remove unlysed cells, and the supernatant was extracted with water saturated phenol and then with chloroform-isoamyl alcohol (24/1 by volume). The nucleic acids were precipitated from the aqueous phase by adding sodium acetate to 0.3 M and 1 volume of 2-propanol, followed by centrifugation. The pellet was resuspended in a mixture of 10 mM Tris-HCl/1 mM EDTA at pH 8.0. The DNA was tested for plasmid content by two dimensional gel electrophoresis (Hintermann et al, 1981).

One of the plasmids found was named "pLH1" -- later renamed "pLAB1000" -- and characterized as described below. The pLH1 plasmid was cleaved with XbaI (XbaI has 1 recognition site in pLH1) and ligated to *E. coli* vector pUC18 (Yanisch-Perron et al, 1985) cleaved with XbaI. The ligation mixture was transformed into *E. coli* strain K514 (Zabeau and Stanley, 1982), and the resulting fusion plasmid was named "pUH1.3" (Fig. 4). A restriction map of the pLH1 plasmid was determined, and the entire pLH1 plasmid was sequenced by the Maxam and Gilbert chemical degradation method. The sequence is 3331 basepairs long and has 3 ORFs reading in the same direction. A fourth ORF (87 amino acids), reading in the opposite direction, is found within ORF 1. The DNA sequence of the pLH1 plasmid is shown in Fig. 3a, and a physical map of the pLH1 plasmid, with restriction sites cleaving once or twice in pLH1, is shown in Fig. 3b.

ORF 1 in pLH1 encodes 361 amino acids. It exhibits amino acid homology with an ORF on the pE194 plasmid from *Staphylococcus aureus* (Horinouchi and Weisblum, 1982A), with an ORF from the pT181 plasmid of *S. aureus* (Khan and Novick, 1983), and with the *prot B* gene of pUB110 (McKenzie et al, 1987). Some of the ORFs that are homologous to the ORF 1 of pLH1 are known to encode site-specific recombinases (Gennaro et al, 1987) that act upon a target site located in the promoter of the recombinase gene. As shown in figure 3c-A, the promoter region of pLH1's ORF 1 contains significant homology with the target site of these site-specific recombinases. It is believed therefore that pLH1's ORF 1 is a site-specific recombinase, catalyzing low frequency recombination at the *RS_A* site in the promoter region of ORF 1 (Figure 3c-B). ORF 1 and the *RS_A* site are located on a 1819 basepair *Ava*I-*Sac*I fragment of pLH1 in Fig. 3a.

ORF 2 in pLH1 shows amino acid homology with the ORF coding for protein A in plasmid pC194 from *S. aureus* (Horinouchi and Weisblum, 1982B; Alonso et al, 1987; McKenzie et al, 1987), with the *prot A* gene of plasmid pUB110 from *S. aureus*, and with an ORF of plasmid pFTB14 of *Bacillus amyloliquefaciens* (Murai et al, 1987) as shown in Figure 3d-A. These ORFs of other genes are known to encode replication proteins that initiate plasmid replication by nicking plasmids at specific sites. A nicking site was defined in pLH1 by the striking homology (Figure 3d-B) between a pLH1 DNA sequence and the nicking sites of the plasmids exhibiting homology with pLH1's ORF 2. Moreover, as found in the other plasmids, this nicking site is located upstream of the gene encoding the replication protein in a cluster of inverted repeated sequences. ORF 2 and the nicking site are located on a 1512 basepair *Ava*I-*Sac*I fragment of pLH1 between nucleotides 2221 and 710 in Fig. 3a. ORF 2 and the nicking site are both required for DNA replication and plasmid stability of pLH1 in a variety of Gram-positive bacteria (See Example 6). From this and from the direct demonstration of single stranded DNA in plasmid preparations of pLH1, it is believed that pLH1 replicates by a single strand intermediate as is the case for several other Gram-positive plasmids (Ter Riele et al, 1986).

In 66 *Lactobacillus* strains, sequences cross-hybridizing to pLH1 were searched by colony hybridization. *Lactobacillus plantarum* strains 183, 195 and 30 (isolated from grass silage by Radar N.V.) cross-hybridized to pLH1 in Southern blots of restriction-digested DNA, but the cross-hybridizing plasmids had a different restriction pattern and different size from pLH1.

Example 4: Constructing *Lactobacillus* vectors based on the pLH1 replicon (Fig. 4)

Plasmid pVA838 (Macrina et al, 1982) carries the macrolide lincosamide streptogramin B ("MLS") resistance gene, encoding resistance to erythromycin, of pAMB1 (Macrina et al, 1982). The DNA sequence of the pVA838 erythromycin resistance gene was published by Martin et al (1987) and found to be very homologous to the MLS resistance gene from Tn917 (Shaw and Clewell, 1985).

pVA838 was cleaved with *Hind*III and *Cla*I and ligated to pBR322 (Bolivar and Backmann, 1979) which was cleaved with *Hind*III and *Cla*I. The ligation mixture was transformed into *E. coli*, which was plated on LB agar supplemented with 100 µg/ml ampicillin. A plasmid was recovered carrying the β-lactamase and tetracycline resistance genes and the origin of replication from pBR322 joined to the erythromycin resistance gene from pVA838, and the plasmid was named "pERM1".

pERM1 was cleaved with *Eco*RV and *Pvu*II and was religated in low DNA concentration. The ligation mixture was transformed in *E. coli* and was plated on LB agar supplemented with 100 mg/l ampicillin and 200 mg/l erythromycin. In this way, a pERM1 derived plasmid was obtained in which the pBR322 tetracycline resistance gene was deleted, and the plasmid was named "pERM2".

pERM2 was cleaved with *Hind*III, and ligated to pLH1 from Example 3 cleaved with *Hind*III. This produced plasmids "pERM3.1" and "pERM3.2" which differ in the orientation of the pLH1 fragment.

pERM3.2 was cleaved with *Eco*RI and *Dde*I, the cohesive ends were made blunt by filling in with Klenow DNA polymerase, and ligated to plasmid pLK58 (Botterman and Zabeau, 1987), cleaved with *Eco*RV. A plasmid was selected containing the erythromycin resistance gene of pERM3.2 inserted in pLK58, and this was named "pGI401". The *P_L* promoter from pLK58 was removed from pGI401 by cleaving pGI401 with *Sal*I and *Stu*I, filling in the sticky ends with Klenow polymerase, and religating the mixture in dilute conditions, yielding plasmid "pGI4010".

pGI4010 was cleaved with *Bam*HI, filled in with Klenow polymerase, then cleaved with *Xba*I, and ligated to plasmid pUH1.3 from Example 3, cleaved with *Sac*I, rendered blunt by the exonuclease activity of Klenow polymerase, and then cleaved with *Xba*I. A plasmid containing pGI4010 as well as the pLH1 *Xba*I-*Sac*I fragment was selected after transformation and plating on LB plates containing 100 mg/l ampicillin and 200 mg/l erythromycin, and was named "pGI4011". The *Bam*HI site was regenerated at the *Bam*HI-*Sac*I fusion.

Example 5: Introducing plasmids from Example 4 into strains of *Lactobacillus* and other Gram-positive bacteria

Lactobacillus plantarum 80, isolated from grass silage by Radar N.V., is noted for its rapid acid production, high acid resistance, high growth rate, homofermentative growth in silage and survival after lyophilization. Several methods can be used to transform this strain with pLH1 derived plasmids. Electroporation, as described below, is particularly efficient.

An overnight saturated culture of *L. plantarum* 80 was diluted 100 fold in MRS medium prewarmed at 37°C and grown in an aerobic shaking culture in MRS medium at 37°C. When the exponential culture reached an optical density ("O.D.") of 0.2-0.8 at 600 nm, its cells were centrifuged for 10 minutes at 5,000 g, resuspended in 1/20 of the original culture volume of sterile distilled water and centrifuged for 10 minutes at 10,000g. They

were then resuspended in 1/100 culture volume of an autoclaved solution of 30% (weight by volume) polyethylene glycol 100 (Merck Schuchardt, Munich, Federal Republic of Germany) in distilled water. All centrifugations and washings were performed at room temperature (25°C). After these manipulations were completed, the cells were put on ice.

For each transformation of *L. plantarum* 80 cells by electroporation with a plasmid from Example 4, 0.8 ml of cell suspension was mixed with 4.0 µg of the vector as a 0.3 µg/ml solution in 10 mM Tris-HCl/1 mM EDTA at pH 8.0, and placed in a 4 mm cuvette in a BioRad Gene Pulser (Bio Rad Chemical Division, 1414 Harbour Way, South Richmond, California 94804, U.S.A.) Plasmid DNA was introduced in *L. plantarum* 80 cells by discharging a single pulse of 1700 volts, capacity of 25 µF, over the cuvette. Typically, time constants of 15 - 20

milliseconds were observed (Time constant is the decay time from peak voltage to 1/e X peak voltage). After the pulse, the cells were left on ice for 30 minutes in the original electroporation cuvette. Then, 10 ml of MRS were added, and the cells were incubated 1 to 2 hours at 28°C. 100 µl of cells (approximately 10⁸ to 10⁹ cells having received 0.05 µg of DNA) were plated on MRS medium supplemented with the appropriate antibiotic: either 10 mg/l chloramphenicol or 10 mg/l of erythromycin. The cells were incubated 48 hours at 37°C. 100 mg/l of lincomycin (Sigma) was also added to the MRS medium before incubation because most erythromycin resistance genes used in this Example, especially the erythromycin resistance gene from pVA838, also induce resistance to lincomycin. Moreover, the addition of lincomycin reduced the frequency of spontaneous erythromycin-resistant mutants of *L. plantarum* 80 from 10⁻⁷ down to less than 10⁻⁹. By this procedure, 10⁶ transformant colonies of *L. plantarum* 80 per microgram of input DNA were obtained.

Using this procedure, high efficiency transformation of *L. plantarum* 50 (another bacterium isolated from silage by RADAR N.V., and noted for its qualities as a silage starter strain), *Lactobacillus casei* strain DSM20020 (on deposit at the DSM under accession no. 20020), *Enterococcus faecalis* strain OG1X (Ike et al, 1983), *Bacillus subtilis* strains 1A40 and 1A42 (from Bacillus Genetic Stock Center, Columbus, Ohio, USA), *B. thuringiensis* strains kurstaki DIPEL (from Abbott Laboratories, Abbott Park, North Chicago, Ill, U.S.A.), entomocides (received from Dr. D. Lereclus, Inst. Pasteur, Paris France) and an isolate of berliner 1715 (received from Prof. A. Klier, Inst. Pasteur, Paris, France), *Bacillus cereus* BCT4 (received from B. Carlton, Ecogen, Langhorne, PA, U.S.A.), *Lactococcus lactis* NIZO 22186 (from Nederlands Instituut voor Zuivelonderzoek, Ede, The Netherlands), could be obtained. Optimizations of the electroporation procedure for specific strains were done as described in Chassy and Flickinger (1987) and in the BioRad manual accompanying the Pulse Controller.

Example 6: Studying replication properties of pLH1 in several Gram-positive bacteria

It was found that plasmids (e.g., pGI4011 from Example 4), containing the pLH1's ORF 2 and nicking site (see Example 3) in a 1652 basepair XbaI - SacI fragment of pLH1, can replicate autonomously in Gram-positive bacteria. This was demonstrated for *L. plantarum* 50 and 80, *L. casei* DSM20020, *E. faecalis* strain OG1X, and *B. subtilis* strain 1A42. There was no evidence of autonomous replication of pLH1 in various strains of *E. coli*. Several pLH1-based replicons, which did not contain the intact 1652 basepair XbaI - SacI fragment of pLH1 (with the ORF 2 and the nicking site), were unable to replicate in Gram-positive bacteria.

Several plasmids originating from different Gram-positive bacteria were introduced in *L. plantarum* 80 by electroporation following the procedures of Example 5. The comparison plasmids were: pVA838 (Macrina et al, 1982), pSA3 (Dao and Feretti, 1985), pTG402 (Zukowski et al, 1983), pTV8 (Youngman, 1987), pDB64 (Gryczan et al, 1980) and pGKV2 (Kok et al, 1985). *L. plantarum* 80 cells transformed with the comparison plasmids and with pERM3.1 (from Example 4) were grown for 13 generations on MRS medium (Oxoid Manual, 1982) without antibiotic pressure. The persistence of the transformed plasmid DNA in such non-selective growth conditions was verified by plating cultures on MRS agar (Oxoid Manual, 1982) supplemented with 10 mg/l erythromycin or chloramphenicol (as appropriate) before and after the non-selective growth for 13 generations. Only pLH1 was maintained in more than 30% of the cells after the nonselective growth for 13 generations. The other plasmids were lost in more than 90% of the cells after non-selective growth for 13 generations. This demonstrated the superiority of pLH1 as a cloning vehicle in *Lactobacillus*.

Plasmids replicating via single strand intermediates like pLH1 (from Example 3) are prone to reorganizations and deletions when used in recombinant DNA constructions (Noirot et al, 1987). No instability was observed in any of the cloning experiments with pLH1. In this regard, during the plasmid transformations in Examples 3, 4, 7 and 8, it was verified by restriction digests of DNA from plasmid minipreparations of transformed bacteria (Anderson & McKay, 1983) that the native configuration of the pLH1 plasmid did not change.

Example 7: Expressing an amylase gene and an endoglucanase gene in *L. plantarum* 80 using vectors based on pLH1 (Figs. 5 and 6)

α-amylase and endoglucanase genes were inserted in separate vectors replicating in *L. plantarum* 80 and assayed for expression as follows.

Plasmid pSTUC (Example 1) was cleaved with SnaBI and DraI, and ligated to pERM3.2 cleaved with SpeI and made blunt end by filling with Klenow polymerase. Two recombinant plasmids carrying the pSTUC fragment with the amylase gene inserted in both orientations in the pERM3.2 replicon were selected by their amylase production (as evidenced by the assay described in Example 1) and erythromycin 200 mg/l resistance in *E. coli*. They were named "pRAM11", and "pRAM12", respectively (Figure 5). Upon introduction in *L. plantarum* 80, both pRAM11 and pRAM12 replicate stably under erythromycin selective pressure, express amylase and

secrete the enzyme into the medium (as evidenced by staining the MRS agar plates containing 1% soluble starch with iodine vapor). Since both pRAM11 and pRAM12 produce similar amounts of amylase activity, the expression of the amylase gene must have been directed from the native (*B. stearothermophilus*) promoter. The amylase expression appears not to have been repressed by presence of glucose in the medium, and not to have been dependent on the growth phase of the strain.

Plasmid pWS11, described by Schwarz et al (1986) and obtained from these authors, carries the *Clostridium thermocellum* endoglucanase *cel A* gene and surrounding sequences including the promoter on a 3.2 Kb *HindIII* fragment. The sequence of this gene is described in Beguin et al (1985). This gene has an endoglucanase activity towards carboxymethyl cellulose ("CMC") and towards lichenan which is a β -1-3-glucan (Hazlewood et al, 1988).

pWS11 was cleaved with *HindIII*, the cohesive ends were filled in by Klenow polymerase, and ligated to plasmid pSA3 (Dao and Feretti, 1985) cleaved with *EcoRV*. Plasmids having the *cel A* gene inserted in the pSA3 *EcoRV* site of pSA3 were identified among transformants in *E. coli*, plated on LB agar supplemented with chloramphenicol 25 mg/l, by: replica plating several colonies on LB agar containing chloramphenicol 25 mg/l and 0.5 g/l CMC (Sigma, low viscosity); allowing these replica plates to grow overnight at 37°C; and then flooding them with a solution of 10 g/l Congo Red (Merck, Darmstadt, Federal Republic of Germany) in water for 15 min at room temperature. The Congo red solution was then poured off, and the plates were washed with 1 M NaCl. Colonies producing endoglucanase were surrounded by a yellow halo on a red background (Cornet et al, 1983A). Two plasmids carrying the endoglucanase in a different orientation were identified, and named "pST11" and "pST12" (Fig. 6). Upon introduction in *L. plantarum* 80, both pST11 and pST12 replicated stably under erythromycin (10 mg/l) selective pressure, and secreted endoglucanase enzyme into the medium. Expression was not repressed by glucose in the culture medium. Endoglucanase activity could also be demonstrated by: incubating supernatants of liquid cultures of the transformed *L. plantarum* 80 containing pST11 or pST12 in wells of agar plates containing 1g/l CMC; measuring the viscosity decrease of aqueous CMC solutions upon incubation with said culture supernatants as described by Cornet et al (1983B); or growing the *L. plantarum* 80 containing pST11 or pST12 in MRS medium from which the normal glucose constituent was omitted, incubating the culture supernatant with a solution of CMC, and measuring the reducing sugars by the method of Somogyi-Nelson (Nelson, 1952). Since both pST11 and pST12 produced similar amounts of endoglucanase activity, the expression of the endoglucanase gene must have been directed from the native *C. thermocellum* promoter.

The stability of the plasmids pST11, pST12, pRAM11, pRAM12 was good with selective antibiotic (10 mg/l erythromycin) pressure. Without such selective pressure, the plasmids were gradually lost in growing cultures as described in Example 26.

Example 8: Constructing a pLH1 derived expression vector (Fig. 7)

A promoter sequence was isolated from *L. hilgardii* 67 by digesting *L. hilgardii* 67 total cellular DNA (prepared according to Example 3) with *Sau3AI* and ligating the fragments in vector pPGV5 cleaved with *BamHI* (Fig. 7b). pPGV5 was derived from pPGV2 (D'Haese et al, 1984) by modifying the polylinker sequence in front of its promoterless CAT86 gene (*Cm^R*) (Fig. 7b). The ligation mixture was transformed in *Bacillus subtilis* strain 1A40, and the recombinants were selected for chloramphenicol resistance. The DNA sequence of one particular *Sau3AI* fragment, inducing very high levels of chloramphenicol acetyl transferase, is shown in Figure 7a. The fragment was named the "PRLB promoter". The pPGV5 vector with this *Sau3AI* fragment was named pPGV1LH (Platteeuw, 1986) and could also transform *L. plantarum* 80 to provide chloramphenicol resistance.

Plasmid pC1, obtained from Dr. Julio Polaina (Instituto de Agroquímica y Tecnológica de Alimentos, Jaime Roig 11, Valencia, Spain) is described in Polaina et al (1988) and its restriction map is shown in Figure 7c. *E. coli*, transformed with this plasmid, express the *Clostridium cellulolyticum* endoglucanase as an intracellular enzyme. This endoglucanase can be demonstrated on LB agar plates containing 1g/l CMC after the colonies have been lysed with chloroform vapor. The endoglucanase gene from *Clostridium cellulolyticum* was excised as a *HindIII* - *Sall* restriction fragment, made blunt end with Klenow, and was ligated to plasmid pGI4011 (from Example 4) which was cleaved with *Sall* and made blunt end with Klenow. The resulting plasmids were named "pGC11" and "pGC12". The structure of pGC11 and pGC12 was identical except for the orientation of the endoglucanase gene (Figure 7c). pGC11 and pGC12 produced small amounts of non-secreted endoglucanase in *E. coli* but no detectable intracellular or extracellular endoglucanase activity when introduced in *L. plantarum* 80 by the electroporation procedure of Example 5.

By inserting the PRLB promoter in pGC11 and pGC12, upstream from the *C. cellulolyticum* endoglucanase gene, the expression of the gene can be induced in *L. plantarum* 80. In this regard, endoglucanase activity can be detected by the methods described in Example 7.

Example 9: Transforming *L. plantarum* 80 by integration of DNA sequences in the chromosome (Fig. 8)

Using the procedure described for *Bacillus subtilis* by O'Kane et al (1986) and the references cited therein, heterologous DNA was integrated in the chromosome of *L. plantarum* 80 by a single recombination event in a region of homology between a plasmid and the chromosome.

Total cellular DNA was prepared from *L. plantarum* 80 as described for *L. hilgardii* in Example 3. This DNA was cleaved partially with *Sau3AI*, so as to produce restriction fragments with an average size of 3 kilobase pairs (verified on agarose gel). These fragments were ligated in plasmid vector pGI4010 (from Example 4),

which was cleaved with BamHI, and the ligation mixture was transformed in *E. coli*. Minipreparation plasmid DNA was prepared from 48 transformants, and the presence of the chromosomal DNA insert was verified by restriction digests. The plasmid DNAs were combined in 8 pools of 6 plasmids each and were introduced in *L. plantarum* 80 by electroporation as in Example 5. About 10 transformants per μg of DNA were obtained with each of the pools. pERM3.2 was introduced in the same batch of *L. plantarum* 80 cells at a frequency of 5×10^5 transformants per μg of plasmid DNA. Electroporation with pure pGI4010 yielded no transformants. Since 8 independent pools gave rise to transformants, it appears that at least 8 (and probably more than 1 out of each 6) random 3 Kbp *Sau*3AI fragments of *L. plantarum* 80 DNA can be used as homology regions mediating chromosomal insertion of pGI4010 derived vector sequences.

From 20 *L. plantarum* 80 colonies, transformed with pGI4010 carrying a chromosomal *Sau*3AI insertion and named "LpGI1" to "LpGI20", plasmid DNA was prepared as described by Anderson and McKay (1983), and total cellular DNA was prepared as described in Example 3. Southern hybridization showed the absence of pGI4010 related sequences in the plasmid fraction except in one transformant. This DNA was digested with restriction enzyme *Eco*RI, separated on an agarose gel, transferred to a nylon membrane (HyBond N, Amersham), and hybridized with radioactively labeled pGI4010 plasmid. The hybridization showed the absence of pGI4010 in the plasmid fraction of the LpGI transformants and in *L. plantarum* 80 and the presence of pGI4010-related material in the digested chromosomal DNA in 19 out of 20 transformants. In one transformant, the LpGI4010 sequences had integrated in the indigenous plasmid of *L. plantarum* 80. The intensity of hybridization to the chromosomal DNA was higher than expected from a single copy insertion and varied in a way unlinked to the amount of chromosomal DNA loaded on the gel. Moreover, the pattern was more complex than expected for a single copy insertion. Therefore, the LpGI strains may carry multiple insertions of pGI4010 in the chromosome.

An *Eco*RI digest was made from total cellular DNA of LpGI2, LpGI4 and LpGI7 and from wild type *L. plantarum* 80. This DNA was diluted to low concentration and ligated, and transformed in *E. coli*. No ampicillin-resistant transformants were obtained with the ligation mixture from *L. plantarum* 80. Ligations of LpGI1 to LpGI20, however, gave rise to ampicillin-resistant *E. coli* colonies. Plasmid DNA from these colonies showed the restriction pattern of the large *Eco*RI - *Bam*HI fragment of pGI4010, linked at the *Bam*HI site to a variable fragment of chromosomal *L. plantarum* 80 DNA. This shows that at least part of the integrated copies of pGI4010 show no major rearrangements.

From a comparison of restriction patterns of the pGI4010 plasmids, carrying random *Sau*3AI fragments of *L. plantarum* 80 DNA, with the plasmids obtained from selecting ampicillin resistance among circularized fragments of LpGI1 to LpGI20, it was possible to identify the plasmid that became inserted in LpGI4. This plasmid was named "pH421", and a concise restriction map of it is given in Figure 8. pH421 has a 3.2Kbp homology region with the *L. plantarum* 80 chromosome.

It was investigated whether the integrated DNA in LpGI strains is stably inherited and whether it influences the phenotype of the host strain. LpGI1 to LpGI20 were mixed with wild type *L. plantarum* 80 cells at a ratio of 1 LpGI cell per 9 *L. plantarum* 80 cells, and the mixture was allowed to grow for 13 generations in MRS liquid medium without any selective pressure. Appropriate dilutions of the resulting culture were plated on MRS agar and on MRS agar supplemented with 10mg/l erythromycin and 100mg/l lincomycin. It was found that 17 out of 20 LpGI strains have the same growth rate as the wild type *L. plantarum* 80 in this assay, and three strains, LpGI5, LpGI10, and LpGI20, do not grow as fast as *L. plantarum* 80.

LpGI2, LpGI4, and LpGI5 were mixed with *L. plantarum* 80 (wild type) at a ratio 1:10, and the mixture was inoculated at 10^6 CFU per gram in autoclaved Italian Rye grass. As in the above experiment, LpGI2 and LpGI4 had the same growth rate as *L. plantarum* 80, whereas LpGI5 grew slower. The ratio of 1/10 was maintained for over 5 days, indicating that the survival of LpGI2 and LpGI4 in grass was not affected.

LpGI2, LpGI4, and LpGI5 were grown for 78 generations in MRS liquid medium without any selective pressure for maintenance of the pGI4010 sequences. After growth for 13, 26, 39, 52, 65 and 78 generations, aliquots of the cultures were plated on MRS agar and on MRS agar supplemented with 10 mg/l erythromycin and 100 mg/l lincomycin. 100 colonies, that appeared on the MRS agar plate, were replicated on MRS agar supplemented with 10mg/l erythromycin and 100mg/l lincomycin. In this assay, more than 95% of the cells of LpGI2 and LpGI4 maintained their erythromycin resistance over 78 generations. However, in LpGI5, erythromycin sensitive cells appeared after 39 generations, and hardly any erythromycin resistant cells (less than 5%) could be found after 65 generations. Thus, integrated sequences are stable in many transformants (e.g., LpGI2 and LpGI4) but not in all transformants (e.g., LpGI5).

The fermentative abilities of LpGI1 to LpGI20 for 49 carbohydrates were tested by means of an API CH50L test) (API System S.A., La Balme les Grottes, 38390 Montalieu Vercieu, France). No differences were found between the fermentative abilities of any LpGI strain and *L. plantarum* 80.

The pattern of total cellular soluble proteins of the LpGI1 to LpGI20 strains and the *L. plantarum* 80 strain was compared by electrophoresis on SDS-PAGE (SDS - Polyacrylamide gel electrophoresis) (Lambert et al, 1987). No significant differences were found between the strains.

This experiment demonstrated the feasibility of stably integrating exogenous DNA in the chromosome of *L. plantarum* 80 without detectably modifying the phenotype of the strain. Particularly, this experiment showed that strain LpGI4 is generated by integration of plasmid pH421 in the chromosome of *L. plantarum* 80 and that LpGI4 has the same phenotype as *L. plantarum* 80 and has a stable integration of pGI4010 sequences. pH421 is therefore used in the following examples as a vehicle for inserting an amylase gene and an endoglucanase

gene in the chromosome of *L. plantarum* 80.

Example 10: Transforming *L. plantarum* 80 by integrating amylase and endoglucanase genes in the chromosome (Fig. 9)

Plasmid pSTUC (from Example 1) was cleaved with *Sna*BI and *Dra*I and ligated with plasmid pJB66 (Botterman and Zabeau, 1987) cleaved with *Eco*RV. Two hybrid plasmids carrying the 1.9 Kbp pSTUC fragment with the amylase gene as an insertion in opposed orientations in pJB66 were selected by screening for amylase activity and restriction mapping of plasmid minipreps from candidate transformants. The plasmids were called "pSATE101" and "pSATE102" (Fig. 9).

Plasmid pWS11 (Schwarz et al, 1986) was cleaved with *Hind*III, the cohesive ends were made blunt by filling in with Klenow polymerase, and the digest was ligated to pSATE101 cleaved with *Sma*I. A plasmid was selected that had the 3.2 Kbp *Hind*III fragment with the *cel* A gene of pWS11 inserted in the *Sma*I site of pSATE101. This plasmid was named "pSATE123". An analogous construction was made for pSATE102 and was named "pSATE125". The direction of transcription of the *cel* A gene and the amylase gene was parallel in pSATE123 and antiparallel in pSATE125 (Figure 9). Both plasmids expressed amylase and endoglucanase activity in *E. coli* as detected by the assays described in Example 7. Moreover, both the amylase and the endoglucanase genes could be excised as a single *Bgl*II fragment.

pSATE123 was cleaved with *Bgl*II, and the cohesive ends were made blunt ended by filling in with Klenow polymerase. The resulting fragments were ligated to plasmid pH421 (from Example 9) which was cleaved with *Sma*I. Two plasmids were selected which had the amylase - endoglucanase cassette integrated in the *Sma*I site of pH421. They were named "pSATE253" and "pSATE256", and the orientations of the respective genes are shown in Figure 9.

Plasmid DNA of pSATE253 and pSATE256 was introduced in *L. plantarum* 80 by electroporation as described in Example 5, and selected on MRS agar containing 10mg/l erythromycin and 100mg/l lincomycin. About 3 transformants per μ g were obtained with each plasmid. In the same electroporation experiment, pERM3.2 was introduced in *L. plantarum* 80 at a frequency of 5×10^6 transformants per μ g plasmid DNA.

8 transformants of *L. plantarum* 80 with pSATE253 were named "LpGA3.1" - "LpGA3.10" (LpGA3.7 and LpGA3.8 were not allocated) and selected for further study. 11 transformants of *L. plantarum* 80 with pSATE256 were named "LpGA6.1" - "LpGA6.11" and selected for further study. All of these LpGA strains (except LpGA6.2) expressed both amylase and endoglucanase activity as determined by the methods described in Examples 1 and 7. One of the LpGA strains, LpGA 3.9, which expressed such activity, was deposited with the DSM.

Undigested chromosomal DNA of each LpGA strain was run on an agarose gel, blotted on Hybond N paper and hybridized with pSATE253. In all strains, except LpGA6.2, hybridization occurred with chromosomal DNA. LpGA6.2 hybridized with an episomal factor, and this clone probably resulted from a non-homologous insertion of pSATE256 in the indigenous *L. plantarum* 80 plasmid. As for the LpGI strains of Example 9, the fermentation patterns of LpGA 6.3, 3.9 and 6.6 (as determined by the API-CH50L test is identical to *L. plantarum* 80, except for the positive reaction on starch of the LpGA strains. Also, the SDS-PAGE of soluble cell proteins reveals no difference between the wild-type and the transformed strains.

Example 11: Constructing a *Lactobacillus* transposon vector ("pTnAMYL") carrying an α -amylase gene (Fig. 10)

Plasmid pJH1 from *E. faecalis* (Banai and Le Blanc, 1983) was cleaved partially with restriction enzyme *Dra*I and ligated to plasmid pLK58 (Botterman, 1986) cleaved with *Sma*I. The ligation mixture was transformed into *E. coli* strain K12 Δ H Δ 1 *trp* (Bernard et al, 1979), and the transformed cells were plated on LB agar supplemented with 10 μ g/ml tetracycline. A transformant was recovered that had the pJH1 tetracycline resistance gene inserted in the pLK58 *Sma*I site, and the transformant was named "pLKTJ". The DNA sequence of the pJH1 tetracycline resistance gene was determined and found to be very homologous to the sequence of the tetracycline resistance gene of plasmid pTHT15 from *Bacillus stearothermophilus* (Hoshino et al, 1985). pLKTJ was cleaved with *Sna*BI and *Eco*RI and was ligated to pERM2 (from Example 4) cleaved with *Hind*III filled in with Klenow polymerase and *Eco*RI. The ligation mixture was transformed in *E. coli* strain K514 (Zabeau and Stanley, 1982), and cells were plated on LB agar supplemented with 10 μ g/ml tetracycline. A plasmid was recovered that carries the pJH1 tetracycline resistance gene linked to the pERM2 replication origin and β -lactamase gene, and the plasmid was named "pTET1".

pTET1 is cleaved with restriction enzymes *Eco*RI and *Pst*I, is ligated to plasmid pTV8 (Youngman, 1987) cleaved with *Eco*RI and *Pst*I, and is transformed into *E. coli* strain HB101. Cells are plated on LB agar supplemented with 10 μ g/ml tetracycline. A plasmid carrying the Tn917 element from PTV8 and the tetracycline resistance gene and gram negative origin of replication from pTET1 is recovered, and the plasmid is named "pTn917.1".

Plasmid pLH1 (from Example 3) is linearized by cleavage with *Hind*III, the cohesive *Hind*III ends are filled in with the Klenow fragment of *E. coli* DNA polymerase I, and the linearized plasmid is then ligated to pTn917.1 cleaved with *Fsp*I.

The ligation mixture is transformed into *E. coli* and is plated on LB agar supplemented with 10 μ g/ml tetracycline. A plasmid is recovered in which both parental plasmids are joined, and the plasmid is named "pTn917.4".

pTn917.4 is cleaved with restriction endonuclease SmaI and is ligated to pSTUC (from Example 1) cleaved with SnaBI and DraI.

The ligation mixture is transformed into *E. coli* and plated on LB agar supplemented with 10 µg/ml tetracycline. Transformants are replicated on LB plates, on which 10 µg/ml tetracycline and 1% soluble starch were added, and were tested for secreted amylase activity by incubating them briefly with iodine vapor. A plasmid is recovered carrying the α -amylase gene inserted into the SmaI site of the Tn917 counterpart of pTn917.4 and exhibiting amylase activity. The α -amylase gene is oriented with its direction of transcription pointing from the inverted repeat of Tn917 towards the erythromycin gene. This plasmid is named "pTnAMYL".

Example 12: Introducing the pTnAMYL Transposon Vector carrying an α -amylase gene into two strains of *Lactobacillus plantarum*

L. plantarum 50 and 80 are transformed with pTnAMYL using the procedure described in Example 5.

100 colonies, exhibiting resistance to erythromycin are replicated onto MRS agar supplemented with 1% soluble starch, 50 µg/ml tetracycline and 10 µg/ml erythromycin. Colonies producing amylase are identified by staining the agar plates with iodine vapor. The native configuration of pTnAMYL in these cells is verified by restriction digests on DNA from plasmid minipreparations (Anderson and McKay, 1983).

Example 13: Transforming *L. plantarum* with an α -amylase gene by integrating the pTnAMYL Transposon Vector in the chromosome of *L. plantarum*

20 colonies of cells of each strain from Example 12, exhibiting amylase activity, are purified and are inoculated in 1 ml of MRS liquid medium with 10 µg/ml erythromycin.

After overnight incubation at 37°C, each culture is diluted to 20 ml with MRS, nalidixic acid is added to 1.0 µg/ml, novobiocin is added to 0.1 µg/ml and erythromycin is added to 10 µg/ml (Novobiocin and nalidixic acid in the above concentrations cure lactobacilli from pLH1).

After 6 hours of growth at 37°C, 0.1 ml of each culture is taken, and 10 ml of MRS (supplemented with erythromycin, novobiocin and nalidixic acid as above) is added. When each culture reaches saturation, 10 µg/ml aliquots are plated on MRS agar with 10 µg/ml erythromycin. The derivatives that are cured of pTnAMYL are identified among the growing colonies by colony hybridization with ³²P radioactively labeled pLH1 DNA as a probe. 20 colonies which show no hybridization to pLH1 are tested and are found to be sensitive to tetracycline and resistant to erythromycin. These colonies are purified, and DNA is prepared from liquid cultures as in Example 3.

An Aval restriction digest of this DNA is size separated on agarose gels and is then transferred to nylon membranes by Southern blotting. The membranes are hybridized to ³²P radioactively labeled Tn917 DNA to verify insertion of the unmodified pTnAMYL construct in multiple sites of the *L. plantarum* genome. The erythromycin resistant, tetracycline insertion mutants exhibit α -amylase activity on starch plates.

Example 14: Transforming *L. plantarum* with the amylase gene from *B. thuringiensis*

Following the procedure set forth in Example 11, pJAM23 (from Example 2) is cleaved with restriction endonucleases PvuII and HindIII, and the cohesive ends are filled in with DNA polymerase I Klenow fragment. The resulting fragments are ligated to pTn917.4 cleaved with SmaI. After transformation into *E. coli* strain HB101, a tetracycline resistant colony exhibiting amylase activity is selected, and its plasmid is named "pTnAMYL2". As with pTnAMYL of Example 11, the direction of transcription of pTnAMYL2 points from the inverted repeat of Tn917 towards the erythromycin gene.

pTnAMYL2 is introduced into *L. plantarum* strains 50 and 80 following the procedure set forth in Examples 5 and 12 and is integrated into their chromosomes as described in Example 13.

Example 15: Constructing a shuttle plasmid ("pV α ") containing an α -amylase gene from *B. stearothermophilus* (Fig. 11)

The vector pVA838 (ATCC catalog number 37160) is able to replicate in *E. coli* and *Streptococcus sanguis* (Macrina et al, 1982) and in *E. faecalis* (Wirth et al, 1986). pVA838 was prepared from the *E. coli* strain DB11 obtained from Dr. Clewell (Clewell, USA) using the rapid LiCl "mini prep" procedure (Wilimzig, 1985). pVA838 was digested with PvuII, and pSTUC (from Example 1) was digested with SnaBI and DraI. The restriction digests were phenol extracted and ligated. The ligation mixture was used to transform *E. coli* (strain tgl, Amersham International) which were spread on LB plates containing 80 µg/ml erythromycin plus 0.2% soluble starch. This permitted the selection and visualization of transformants harboring recombinant plasmids in which the small region between the two PvuII sites of pVA838 was substituted by the SnaBI-DraI fragment from pSTUC containing the α -amylase gene (Fig. 11). Single transformant colonies were checked for the presence of the expected recombinant plasmid by restriction analysis of miniprep plasmid DNA (Wilimzig, 1985). One such transformant, named "pV α ", was retained for use as a shuttle plasmid in the transformation of *E. faecalis* as described below in Example 16.

Example 16: Transforming *E. faecalis* protoplasts with the pV α shuttle plasmid containing an α -amylase gene

The *E. faecalis* OG1X strain (Ike et al, 1983), obtained from Dr. Clewell, was used to prepare protoplasts according to the method of Wirth et al (1986). A 600 ml culture of the OG1X strain in THB (Todd-Hewith broth, made by Oxoid Ltd. Basingstroke, Hampshire, England), supplemented with 2% glycine, was brought to an

O.D. of 0.15 (at 650 nm). Cells were collected by centrifugation and resuspended in 10 ml of THB containing 60% glycerol. Aliquots (1 ml) were frozen at 70°C until they were treated with lysozyme (1 mg/ml) and 10 mg/ml bovine serum albumin ("BSA") in TSCM buffer (Tris-HCl 100 mM, pH 8.0; Na Succinate 500mM; CaCl₂ 40mM; MgCl₂, 10mM) during 120 min. at 37°C to prepare protoplasts. Under these conditions, the efficiency of protoplasting was > 99,99 %, and protoplasts could be regenerated at a frequency > 90%.

The protoplasts were collected by centrifugation (10 min. at 3,000 g), were washed once in 10 ml of TSCM buffer containing 100 µg/ml BSA, and were resuspended by gentle shaking in 1 ml of the same buffer. A 100 µl sample of the protoplasts (approximately 10⁸ cells) was mixed with about 100 µl (about 1 µg) of the pVα shuttle plasmid (from Example 15) in 2 X SSC (1 X SSC is 0.15 M NaCl plus 0.015 M Na-citrate). The mixture was added to 1.8 ml of filter sterilized 40% polyethyleneglycol with a molecular weight of 1000 ("PEG"; Sigma) in TSCM buffer. Mixing of the protoplasts, pVα and PEG was done by inverting five times a tube containing these materials. Then, this tube was incubated for 3 min. at 0°C and then for 5 min. at 37°C. The mixture in the tube was then added to a centrifuge tube containing 10 ml of TSCM buffer, supplemented with BSA (100 µg/ml). Protoplasts, recovered after a 10 minute centrifugation at 12,000 g, were washed once in 10 ml of THB medium containing Na Succinate (135 mg/ml) and BSA (100 mg/ml). The protoplasts were resuspended in 1 ml of the same medium and incubated for 120 min. at 37°C for phenotypic expression of transformed DNA. Finally, 20 to 100 µl samples were plated onto THB agar plates containing (per liter) 8 g of agar, 135 g of Na Succinate, 100 mg BSA and 5 mg erythromycin.

After 2 to 3 days incubation at 37°C, erythromycin resistant colonies were obtained at a frequency of about 10³/µg DNA. They were replicated onto agar THB plates containing 20 µg/ml erythromycin plus 0.2% soluble starch. Erythromycin resistant colonies displayed a clear halo upon exposure to iodine vapor. Their plasmids were further identified by restriction analysis of mini preparation plasmid DNA (Ish-Horowicz and Burke and 1981). As expected, erythromycin-resistant, α-amylase positive colonies harbored the pVα recombinant plasmid. The halo surrounding the colonies on starch plates exposed to iodine vapor indicate that the *B. stearothermophilus* α-amylase gene was actively expressed in *E. faecalis* and that the α-amylase was secreted into the medium.

Example 17: Constructing another shuttle plasmid ("pVAα") from *B. licheniformis* (Fig. 12)

The *B. licheniformis* α-amylase had been cloned from different strains, and its sequence had been determined by several groups (Ortlepp et al, 1983; Sibakov and Palva, 1984; Piggott et al, 1984; Joyet et al, 1984; Stephens et al, 1984; Yuuki et al, 1985; Gray et al, 1986). Plasmid pRPI bearing the α-amylase gene from the *B. licheniformis* RP01 as a BclI fragment (Piggott et al, 1984) was received from Dr. D.J. McConnell, Department of Genetics, Trinity College, Univ. Dublin, Republic of Ireland. This plasmid was restricted with BclI, and the 2.5 kb BclI fragment was recovered from 0.8% agarose gel in TBE buffer after electrophoresis for 2 hours at 75V in a horizontal submarine apparatus filled with TBE buffer. Recovery was performed according to Maxam and Gilbert (1980). This 2.5 kb BclI fragment containing the complete transcription unit for the α-amylase (Piggott et al, 1984) was ligated into pUC18 (Yanisch-Perron et al, 1985) previously cut with BamHI. The ligation mixture was transformed into *E. coli* strain JM101 (Messing et al, 1981). Colonies harboring recombinant plasmids comprising the pUC18 backbone plus the 2.5 kb α-amylase insert were selected on agar plates (LB supplemented with 50µg ampicillin/ml and 0.2% starch). Candidates were identified by the presence of a clear halo surrounding the colony after a brief exposure to iodine vapor. The identity of the recombinant plasmids present in such transformants was checked by restriction analysis performed on plasmid DNA prepared according to the LiCl rapid procedure (Wilimzig, 1985). One transformant colony harboring the expected recombinant plasmid was retained. After single colony purification, it was used for large scale purification of the recombinant plasmid by the alkaline lysis-CsCl method. This plasmid was named "pα21".

Plasmid pVA838 was prepared as before and digested with EcoRI and SphI. In parallel, the plasmid pα21 was digested with the same enzymes. The restriction digests were phenol-extracted and ligated. The ligation mixture was used to transform *E. coli* strain tgl (Amersham). Spreading on LB plates containing 80 µg/ml of erythromycin plus 0.2% starch allowed the selection and visualization of transformants harboring recombinant PVA838 plasmids whose EcoRI-SphI insert coming from pα21 and containing the α-amylase gene. Single transformant colonies were checked for the presence of the expected recombinant plasmid by restriction analysis of rapid DNA preparations as described before. One transformant colony harboring the expected recombinant plasmid was retained. After single colony purification, it was used for small scale purification of the recombinant plasmid according to Wilimzig (1985). This plasmid was named "pVAα" and used for the transformation of *E. faecalis* OG1X protoplasts strain as described in Example 16. After 2 or 3 days of incubation at 37°C, erythromycin-resistant colonies were obtained at a frequency of about 10³/µg DNA. They were replicated onto agar THB plates containing 50 µg erythromycin/ml plus 0.2% soluble starch. All colonies displayed a clear halo upon exposure to iodine vapor. Their plasmids were further identified by restriction analysis of mini-preparation plasmid DNA prepared according to Ish-Horowicz and Burke (1981). As expected, all transformants harbored the pVAα recombinant plasmid. The large halo surrounding the colonies clearly indicated that *B. licheniformis* α-amylase gene was actively expressed in *E. faecalis*, and that the α-amylase protein was released into the medium.

Example 18: Constructing other shuttle plasmids ("pTV α ") containing the α -amylase gene from *B. stearothermophilus* (Fig. 13)

The vector pTV1 can be propagated in *B. subtilis* (Youngman, 1987) or *E. faecalis* (Weaver and Clewell, 1987) as an autoreplicative plasmid but only at low temperatures (e.g., 32°C). At non-permissive temperatures (e.g., 48°C), its origin of replication is no longer functional, and the plasmid is lost in a few generations. However, the erythromycin resistance phenotype can be retained in some of the cells exposed to non-permissive conditions due to the transposition of Tn917 into the chromosome. Thus, pTV1 and its derivatives, such as PTV8 (Youngman, 1987), can be used either as an autoreplicative vector or as an integration vector as used below.

A shuttle plasmid was made in *E. coli*. First, pSTUC (from Example 1) and pBR322 were each digested with PvuII, phenol extracted and were ligated to yield a "pBR α 1" plasmid consisting of a pBR322 backbone plus an α -amylase gene inserted at the PvuII site in the orientation shown in Fig. 13. The size of the pBR α 1 plasmid was reduced by deletion of the HindIII fragment bearing the tetracycline resistance gene, yielding a "pBR α 2" plasmid. The pBR α 2 plasmid was restricted with PvuII, and the plasmid pTV8 was cut with SmaI. These digests were phenol extracted and ligated to yield pBR α 2-pTV8 concatemers which were named "pTV α 1" and "pTV α 2" according to the orientation of the insert as shown in Fig. 13.

Example 19: Transforming *E. faecalis* protoplasts with the pTV α autoreplicative shuttle plasmids containing an α -amylase gene

The two shuttle plasmids pTV α 1 and pTV α 2 (from Example 18) were purified from *E. coli* by the LiCl rapid miniprep method (Wilimzig, 1985) and were used to transform *E. faecalis* strain OGIX protoplasts as described in Example 16. Control experiments with intact pTV8 showed that the origin of replication of this plasmid was functional in the *E. faecalis* strain at the permissive temperature (32°C). Transformation with the pTV α plasmids yielded erythromycin-resistant colonies upon selection at 32°C on THB plates supplemented with 5 μ g/ml erythromycin. These transformants were replicated onto agar THB plates at 32°C supplemented with 20 μ g/ml erythromycin plus 0.2% soluble starch. The erythromycin-resistant colonies displayed a clear halo upon exposure to iodine vapor. Their plasmids were further identified by restriction analysis of miniprep plasmid DNA. Erythromycin resistant, α -amylase positive colonies harbored the pTV α plasmid. This shows that, under permissive conditions, the pTV α plasmids can be maintained in *E. faecalis* as autoreplicative plasmids with their amylase passenger genes being actively expressed.

Example 20: Integrating the pTV α borne α -amylase gene into *E. faecalis* chromosome by Tn917 transposition

Clones of *E. faecalis* strain OGIX with the autoreplicative pTV α 1 and pTV α 2 plasmids (from Example 19) were subjected to the procedure of Youngman (1987). In each case, a culture was prepared in liquid THB medium supplemented with 50 μ g/ml erythromycin. This culture was grown at 32°C with moderate aeration up to stationary phase. The culture was then diluted 1:50 with THB broth containing 0.5 μ g/ml erythromycin. This culture was grown overnight at 45°C with moderate aeration. Then, the culture was diluted again 1:50 with the same medium and grown at 45°C with moderate aeration. Two further cycles of dilution/growth at 45°C were performed. Cells were collected by centrifugation and were resuspended in the same medium to 1/25th of the original volume. Serial dilutions were prepared, plated onto THB plates containing 10 μ g/ml erythromycin and incubated at 28°C. Individual colonies were then replicated using sterile toothpicks onto THB plates containing 50 μ g/ml of erythromycin plus 300 μ g/ml of lincomycin, and 0.2% starch. 14 individual clones were retained in the pTV α 1 and pTV α 2 series, respectively, on the basis of their erythromycin/lincomycin resistance and α -amylase positive phenotype at 28°C. To demonstrate the absence of autoreplicative plasmids, each clone was subjected to the plasmid miniprep procedure of Ish-Horowicz and Burke (1981). Each preparation was analyzed by agarose gel electrophoresis, on the one hand, and used to back-transform *E. coli*, on the other hand. 13 clones from the pTV α 1 series, and 12 clones from the pTV α 2 series were negative by both criteria. These were further characterized as follows.

First, the stability of the phenotypic traits resulting from the expected transposition event was demonstrated by culturing each clone under non-selective conditions, i.e., in liquid THB medium at 37°C, for 2 growth cycles. Dilutions were then made and plated onto THB plates and incubated at 37°C.

200 individual colonies were then toothpicked onto THB plates containing 50 μ g/ml of erythromycin, 300 μ g/ml of lincomycin and 0.1% starch. Upon incubation at 37°C, 12 out of 13 clones from the pTV α 1 series and 12 out of 12 clones from the pTV α 2 series turned out to be 100% stable. These experiments strongly suggested that the genes responsible for the stable phenotypic traits were chromosome-borne, since the same phenotypic traits turned out in control experiments, to be highly unstable under identical experimental conditions when directed by plasmid-borne genes (about 10% phenotypic reversion per generation).

In order to formally demonstrate the chromosomal location of both the erythromycin-resistance and the α -amylase marker genes as a result of transpositional integration, hybridization experiments were performed on several individual clones from each series according to the method of Southern (1975). These experiments clearly demonstrated that these genes were indeed chromosome-borne, the site of transposition being different in several individual clones.

Altogether, it was found that the α -amylase gene had been stably incorporated into the chromosome of *E. faecalis* and expressed as an active enzyme released into the medium, as evidenced by the presence of a halo surrounding the colonies.

By the method of Example 9, the growth rate of *E. faecalis* having pTV α and pV α inserts was found to be

comparable to the growth rate of wild-type *E. faecalis* OGIX.

Example 21: Constructing a set of single recombination prone integration vectors ("pG18 $\alpha\phi$ ") containing the α -amylase gene from *B. stearothermophilus* (Fig. 14)

A plasmid bearing a segment of chromosomal DNA from *E. faecalis* but lacking an origin of replication for that host behaves as an integration vector as a result of a single homologous recombination event taking place between duplicated chromosomal sequences. To make such a vector useful for purposes of this invention, α -amylase and a phenotypic marker, which will be expressed in *E. faecalis*, are inserted in the vector. A similar construction has recently been shown to function as an integration vector in *B. subtilis* (O'Kane et al, 1986).

The erythromycin marker from pVA838 was excised as a HindIII-AvaI fragment and inserted into pUC18 (Yanisch-Perron et al, 1985) cleaved with HindIII and SalI. Transformation into *E. coli* allows the recovery of clones harboring plasmids consisting of the pUC18 backbone plus the erythromycin resistance marker. These clones are selected on LB agar plates containing 50 μ g ampicillin and 80 μ g erythromycin per ml. After single colony purification, they are checked for the presence of the desired recombinant plasmid as described above by restriction analysis of miniprep plasmid DNA (Wilimzig, 1985). One such clone is retained for further work and is named "pG18".

The α -amylase gene is then inserted into pG18. pG18 is restricted with SmaI, and pSTUC (from Example 1) is restricted with PvuII. Restriction digests are phenol extracted and ligated as described above. This ligation mixture is used to transform *E. coli* which allows the recovery of clones harboring the plasmid named "pG18 α ". In addition to being erythromycin and ampicillin resistant, these clones display a clear halo on starch plates exposed to iodine vapor.

In the next step, random fragments of the *E. faecalis* OG1X chromosome are inserted into pG18 α . Chromosomal DNA is prepared according to Dale and Greenaway (Dale and Greenaway, 1984) and is digested with XbaI. In parallel, pG18 α is cut with XbaI, and the digests are treated with calf intestine alkaline phosphatase ("CIP"). CIP treated, XbaI digested pG18 α is then ligated to XbaI chromosomal fragments. The ligation mixture is used to transform *E. coli*, yielding a set of clones harboring recombinant plasmids, named generally "pG18 $\alpha\phi$ ". These clones are selected on LB plates supplemented with 80 μ g erythromycin/ml plus 50 μ g ampicillin/ml and 0.2% starch. Halo positive colonies are individually analyzed by restriction analysis of miniprep plasmid DNA as described above to establish the presence of recombinant plasmids of the expected structure. Among these, a subset of 24 pG18 $\alpha\phi$ clones with no PstI site in the ϕ region is retained for the integration experiments described below. Their plasmids are numbered pG18 $\alpha\phi$ 1 to 24.

Example 22: Integrating the pG18 $\alpha\phi$ borne α -amylase gene into an *E. faecalis* chromosome by single homologous recombination

Miniprep plasmid is prepared from individual cultures of clones harboring plasmids pG18 $\alpha\phi$ 1 to 24 (from Example 21). DNA from each preparation is used as before to transform *E. faecalis* OG1X protoplasts. In some cases, transformants are obtained which have integrated a given pG18 $\alpha\phi$ plasmid into their chromosome as a result of a single recombination event between the region of the plasmid and its chromosomal counterpart. These are identified among erythromycin-resistant halo-positive colonies by Southern blotting experiments as described below. PstI digested total DNA is hybridized with (³²P)-labeled pG18 α as a probe. Control experiments are performed in parallel with total DNA from the corresponding pG18 $\alpha\phi$ transformed *E. coli*. Each control displays only one band upon hybridization with the probe, corresponding in size to the linear form of the pG18 $\alpha\phi$ plasmid-harbored by that clone (Fig. 14). Each integrative transformant displays two bands when hybridized with (³²P)-labeled pG18 α . The sizes of those two bands are identical for all transformants of a given homology region, but different among different homology regions. The same band hybridizes with pUC18 as a probe. These results demonstrate that each pG18 $\alpha\phi$ plasmid has recombined into a different site on the *E. faecalis* chromosome, depending on the nature of the region present in each clone. Again, the presence of a halo surrounding the colonies on starch plates exposed to iodine vapor indicates that the α -amylase gene is expressed.

Example 23: Constructing a set of double recombination prone integration vectors ("pG18 $\phi\alpha$ ") containing the α -amylase gene from *B. stearothermophilus* (Fig. 15)

A plasmid, bearing a segment of chromosomal DNA from *E. faecalis*, into which the α -amylase gene has been inserted, and lacking an origin of replication for that host, behaves as an integration vector. In such a case, as opposed to the mode of integration of the pG18 $\alpha\phi$ vector described in Example 22, a double recombination event can occur, one on each side of the α -amylase gene. In order to allow for the selection of such double recombination prone integrative transformants, a marker gene should be present next to the α -amylase gene inside the chromosomal fragment. Constructions of this kind have been shown to function as integration vectors in *B. subtilis* (Youngman, 1987).

Random fragments of the *E. faecalis* chromosome are inserted into the pUC18 vector as follows. Chromosomal DNA from *E. faecalis* is prepared (Dale and Greenaway, 1984) and digested with NheI. In parallel, pUC18 is digested with XbaI. Restriction digests are phenol extracted and are ligated. Transformation into *E. coli* allows the recovery of plasmids consisting of the pUC18 backbone plus a fragment of *E. faecalis* chromosomal DNA. After single colony purification, they are checked for the presence of the desired recombinant plasmid by restriction analysis of miniprep plasmid DNA (Wilimzig, 1985). The plasmids

present in these clones are given the generic name "pUC18 ϕ ". Among these plasmids, a subset of 24 pUC18 ϕ plasmids are retained having no XbaI site while comprising at least one site for the following blunt-end restriction enzymes: Ball, EcoRV, HpaI, NaeI, NruI, and StuI (labeled "R" in Fig. 15). These plasmids are numbered pUC18 ϕ 1 to 24.

To introduce the α -amylase gene coupled to the erythromycin resistance marker into the pUC18 ϕ 1 to 24 plasmids, the following steps are taken. Each of the plasmids is cut with its cognate blunt-end restriction enzyme as listed above and ligated to PvuII cut pG18 α (from Example 21). Transformation into *E. coli* yields a set of recombinant plasmids, each consisting of a given pUC18 ϕ backbone plus an insert comprising the α -amylase/erythromycin resistance region of pG18 α . The corresponding clones are selected individually on LB plates supplemented with 50 μ g ampicillin/ml plus 80 μ g erythromycin/ml and 0.2% soluble starch. Halo-positive colonies are analyzed by restriction analysis of miniprep plasmid DNA to establish the presence of recombinant plasmids of the expected structure. These plasmids are given the generic name "pG18 $\phi\alpha$ " and numbered pG18 $\phi\alpha$ 1 to 24.

Example 24: Integrating the pG18 $\phi\alpha$ -borne α -amylase gene into an *E. faecalis* chromosome by double homologous recombination

Miniprep plasmid DNA is prepared as described above from individual cultures of clones harboring plasmids pG18 $\phi\alpha$ 1 to 24 (from Example 23). DNA from each preparation is used as before to transform *E. faecalis* protoplasts.

Plating on THB plates containing 5 μ g erythromycin/ml allows, in some cases, the recovery of transformants which have integrated the erythromycin resistance gene and the α -amylase gene into their chromosomes as a result of double recombination events which have occurred between each of the ϕ regions of pG18 $\phi\alpha$ and their chromosomal counterparts. These transformants are erythromycin-resistant and halo-positive and display a specific pattern in Southern blotting experiments as described below. Total DNA is extracted from candidate colonies, is digested with XbaI and is analyzed by Southern blotting using (³²P)-labeled pG18 α as a probe. Control experiments are performed in parallel with total DNA from the corresponding pG18 $\phi\alpha$ transformed *E. coli*. Each control displays one band upon hybridization with the probe, corresponding in size to the linear form of the pG18 $\phi\alpha$ plasmid harbored by that clone (Fig. 15). The expected *E. faecalis* integrative transformants display two bands with the probe with band sizes that vary from one transformant to another. The transformants showing two bands with the probe display an identical pattern in Southern blotting experiments using (³²P)-labeled pV α 1 as a probe, but they do not show any hybridizable band with (³²P)-labeled pUC18 as a probe, unlike the integrative transformants resulting from the single recombination event described above.

When digested with HindIII and analyzed by Southern blotting using pG18 α as probe, the total DNA of transformants, showing two bands with the probe, display a single major band on the autoradiogram. This band corresponds in size with the HindIII fragment from pG18 α spanning the *erm* and α regions (See Fig. 15). These results demonstrate that the erythromycin resistance gene plus the α -amylase gene from pG18 $\phi\alpha$ plasmids have recombined by double cross over into different sites of the *E. faecalis* chromosome, depending on the nature of the region present in a given clone. Again, the halo in the presence of iodine vapor indicates that the α -amylase gene is expressed.

Example 25: Evaluation of the performance of transformed *L. plantarum* 80 and *E. faecalis* OG1X in grass silage

Comparisons have been made between wild type and transformed *L. plantarum* 80 and *E. faecalis* OG1X strains as to the ability to extract fermentable sugars from a variety of commonly used silage materials and silage additives and the rate of acidification in a number of liquid media.

25.1. Production of reducing sugars

The ability to convert poly- or oligosaccharides into lower molecular weight mono- or oligosaccharides upon incubation was measured as an increase in reducing sugar content. The Somogyi-Nelson method (Nelson, 1952) is the most reliable for this purpose (Breuil and Saddler, 1985). A panel of strains of lactic acid bacteria was incubated with a collection of test substrates and additives commonly used in silage.

The strains consisted of:

L. plantarum 80 (hereinafter also referred to as Lp 80)

L. plantarum 80 containing pRAM11 (Example 7) mixed in a 1:1 ratio with *L. plantarum* containing pST11 (Example 7).

LpGA6.6 (Example 10)

E. faecalis OG1X (hereinafter also referred to as EfOG1X)

E. faecalis OG1X with plasmid pV α (Example 17) A blanco (water) was included

The substrates were:

- soluble starch 3% (DIFCO solubilized by boiling and dialyzed).
- carboxy methyl cellulose (CMC) low viscosity 1% (solubilized by boiling and dialyzed).
- corn starch 3% in dialysis buffer and dialyzed.
- milled corn grains 3 % in dialysis buffer.

- blanco: dialysis buffer

The substrates were dialyzed (cutoff 12,000 dalton) overnight for the first time then twice dialyzed for 2 hours against 5 mM CaCl₂, 50 mM Na-Succinate buffer pH 5.8 at 4°C. This removes most of the indigeneous low molecular weight carbohydrates in e.g. corn starch.

The strains were cultured overnight in MRS (for Lp80) or TH (for *E. faecalis*). For transformed strain, 10 µg/ml erythromycin and 100 µg/ml lincomycin were added.

2 ml of Lp80 cultures and 4 ml of *E. faecalis* cultures were inoculated in 100 ml MRS from which the normal glucose constituent was omitted. Again erythromycin and lincomycin were added for the transformed strain. The bacteria were grown for 15 h at 37°C. The OD and pH of each culture was determined. The OD's of all cultures were equalized to an OD₆₀₀ = 0.3, while the pH was brought to 5.8. To kill off the bacteria each culture was incubated for 15 minutes with shaking after addition of 0.1 ml chloroform per 100 ml. Killing the bacteria excludes the consumption of carbohydrates by fermentation. The cultures and substrates were preheated to 60°C and seven ml of each culture was mixed with 10 ml of each substrate. From each mixture a 4 ml sample was frozen at -20°C (this constitutes time = 0). Two more 4 ml samples were incubated at 60°C. For each mixture one of these 4 ml samples was frozen (-20°C) after 3 hours and the other one after 38 hours of incubation.

For analysis the frozen samples were thawed and subsequently centrifuged for 2 minutes in an Eppendorf centrifuge. Reducing sugars were determined on the supernatans by the Somogyi-Nelson method (Nelson, 1952).

The measurement of the OD was roughly linear to the concentration of glucose in a series of controls and 1 OD equalled approximately 0.14 g/l.

The results are presented in Table 1. The figures given in Table 1 are the net increases in glucose equivalents in the medium (in g/l) after 38 hours of incubation.

Table 1

	soluble starch	CMC	Corn starch	Corn	Blanco
Lp80	0.016	0.003	0.001	0	0
Lp80 pRAM11	1.472	0.161	0.252	0.784	0
Lp80 pST11					
LpGA6.6	0.375	0.265	0.033	0.250	0
EfOG1X	0.196	0.012	0	0.083	0
EfOG1X pVAα	0.452	0.009	0.001	0.177	0
Blanco	0.123	0.001	0	0.107	0

Table 1 shows that the mixture of Lp80 transformed with pRAM11 and Lp80 transformed with pST11 and LpGA 6.6 performed better than the controls for all substrates while EfOG1X transformed with pVAα performed better than its control for soluble starch and milled corn grains.

25.2. Rate of acidification

The following strains:

- *L. plantarum* 80 (Lp80)
- LpGI4 (Example 9)
- Lp80 pRAM11 (Example 7)
- Lp80 pST11 (Example 7)
- a mixture of LpGA3.2, 3.1, 6.3, and 6.4 (1:1:1:1) (Example 10)

were grown in MRS, inoculated at a 2% concentration in MRS without glucose, and grown overnight at 37°C. The pH and the O.D.₆₀₀ of the cultures were uniformized to approx. pH = 6.0 and OD₆₀₀ = 0.6 (10⁸ cells/ml). In a 100 ml bottle were mixed:

- 10 ml of MRS without glucose
- 1 ml of one of the above strains
- 1 mg erythromycin for all strains except *L. plantarum* 80
- 5 mg kanamycin
- 2 grams of one substrate from the list below
- sterile water up to 100 ml.

The final concentration of the cells was 10⁶ cells per ml.

The following substrate solutions were used:

- soluble starch: see 24.1
- milled corn: see 24.1
- CMC: see 24.1
- dextrose (positive control)
- no additives as blanco (negative control)

The substrates were not sterilized to avoid modification of the physicochemical structure. However, the probability of secondary infection was reduced by the addition of kanamycin which does not affect Lp80 and erythromycin (10 µg/ml) which does not affect the transformed strains.

The 100 ml bottles as prepared above were incubated at 37°C with shaking. The pH was measured after 0, 2 and 5 days.

The results are presented in Table 2.

Table 2

	starch	corn	CMC	dextr	H ₂ O
Lp80	6.09	4.98	6.42	6.25	5.86
	5.92	5.50	6.41	4.18	5.62
	4.95	5.00	6.10	3.36	5.86
LpGI	6.08	4.87	6.37	4.20	5.70
	6.01	4.85	6.48	3.83	5.70
	5.70	4.89	6.46	3.35	5.70
pRAM11	6.12	4.10	6.32	6.18	5.98
	5.63	3.68	6.30	3.12	5.30
	4.29	3.42	6.22	3.61	5.30
pST11	6.04	4.88	6.23	6.30	5.72
	5.89	4.85	6.10	5.12	5.60
	5.92	4.90	5.60	3.42	5.93
LpGA 3.1-4	6.10	4.88	5.91	6.22	5.14
	6.06	4.71	5.29	3.92	5.07
	4.20	3.75	5.39	3.55	5.30

Table 2 shows that pRAM11 and the LPGA mixture induced a more pronounced pH decrease than the other strains on soluble starch and milled corn. Table 2 also shows that pST11 and the LpGA mixture induced a more pronounced pH decrease than the other strains on CMC. Some variability of the results is probably due to growth of contaminating bacteria.

Example 26: Stability and competitive abilities of engineered *Lactobacillus plantarum* strains in various conditions

26.1. To test the stabilities of the engineered *Lactobacillus plantarum* 80 in the absence of selective pressures on the foreign DNA fragments, bacteria were fermented overnight in MRSE (MRS supplemented with 10 µg/ml erythromycin), pelleted and resuspended in the original volume of MRS. 10 µl of this suspension was used to inoculate 10 ml MRS, and was grown to saturation (overnight, 37°C). 10 µl of this DAY 1 culture was used to inoculate 10 ml MRS and subsequently cultured overnight to obtain a DAY 2 culture. The above procedure was repeated once more to obtain a DAY 3 culture. Aliquots of DAY 1, DAY2 and DAY 3 cultures were taken and the number of viable cell was determined by plating sequential dilutions on MRS and MRSE agar.

The number of colony forming units (CFU) of transformed Lp80 on both MRS and MRSE after 1, 2, and 3 days (respectively 13, 26 and 39 generations) are presented in Table 3. The numbers in Table 3 are in 10⁷ CFU per ml. A comparison of the figures in Table 3 for MRS and MRSE shows the stability of the fermented transformed strains under non-selective conditions.

Table 3

Segregation of engineered DNA, monitored as erythromycin resistance, upon non-selective growth of transformed *L. plantarum* 80

	DAG1		DAG 2		DAG 3	
	MRS	MRSE	MRS	MRSE	MRS	MRSE
pST11	152	26	242	10	232	-
pRAM11	34	26	198	2	186	-
1PGA3.1	142	115	236	236	260	179
1PGA3.2	152	179	192	284	305	186
1PGA3.3	206	181	209	234	176	130
1PGA3.4	162	153	256	315	183	161
1PGA3.6	239	186	238	278	185	156
1PGA3.9	184	167	264	276	227	216

Table 3 (continued)

	DAG1		DAG 2		DAG 3	
	MRS	MRSE	MRS	MRSE	MRS	MRSE
10GA6.1	119	128	191	226	127	80
1PGA6.2	83	60	273	47	186	8
1PGA6.3	84	94	249	231	132	151
1PGA6.4	195	139	209	291	162	129
1PGA6.5	180	149	271	242	182	169
1PGA6.6	103	121	210	182	177	46
1PGA6.7	155	129	289	260	149	114
1PGA6.8	158	131	287	228	213	136
1PGA6.9	137	120	221	206	127	162
1PGA6.11	124	98	240	260	143	98

It is clear that under these non-selective conditions the erythromycin resistance, when contained on plasmids (see also Example 6), is rapidly lost, while the same characteristic is very stable when present in the bacteria as a chromosomal insert. Only in 1 case (LpGA 6.2) the resistance is lost after 3 days possibly caused by the presence of the erythromycin resistance gene on an indigenous plasmid of Lp80 (see Example 10).

26.2. Experiments to test the abilities of the engineered strains to compete with the wild type strains were performed as follows.

About 250 g of fresh grass was autoclaved and inoculated with 10^8 cells of wild type *L. plantarum* and 10^7 cells of LpGI2 (or analogously LpGI4 or LpGI5) and stored under conditions mimicking silage (anaerobic, 28°C). Cells are taken from overnight cultures (37°C). After respectively 1, 2, 3, 4, 5 and 11 days, 25g of grass from each culture was suspended in 225 ml 0.1 % proteose pepton, serially diluted to a factor of 10^{-6} and plated on both MRS and MRSE. The ratio of the number of CFU's observed on MRSE to the number observed on MRS is indicative of the competitive abilities of the strain containing the erythromycin resistance on a chromosomal insert as compared to the wild type strain.

The results are presented in Table 4. The numbers in Table 4 refer to 10^6 CFU per gram of ensiled grass. It is obvious from Table 4 that LpGI2 and LpGI4 have the same growth rate and survive equally well in grass silage as wild type *L. plantarum* 80. As in liquid culture (Example 9), LpGI5 has a lower growth rate although its survival is comparable to wild type *L. plantarum* 80. This is probably due to the fact that insertion of the Em gene occurred in a vital place of the bacterial chromosome, proving the importance of an adequate choice of a chromosomal segment as a recombination target.

Table 4

Comparison between wild-type and transformed
L. plantarum 80 as to growth rate and subsequent
survival in ensiled grass

Lp80 + LpG12 (1:9)

Day	MRS	MRSE	Ratio MRS/ MRSE
1	288	45	1/6
2	313	78	1/4
3	220	56	1/4
4	65	19	1/3
5	85	15	1/6
11	3	1	1/3

Lp80 + LpG14 (1:9)

Day	MRS	MRSE	Ratio MRS/ MRSE
1	136	40	1/3
2	800	86	1/9
3	275	44	1/6
4	274	29	1/9
5	53	14	1/4
11	7	1	1/7

Lp80 + LpG15 (1:9)

Day	MRS	MRSE	Ratio MRS/ MRSE
1	326	4	1/81
2	132	2	1/66
3	245	3	1/61
4	28	0.1	1/282
5	36	0.6	1/61
11	5	.02	1/250

In an analogous experiment, insertion of Lp80 with respectively LPGA6.3, LPGA6.6 and LPGA1 3.9 (all strains containing the amylase- endoglucanase-erythromycin resistance cassette as a chromosomal insert), in a ratio of 1:9, were fermented in 10 ml MRS during 1 day.

For each culture a sample of 100 µl was taken, serially diluted to a factor 10⁻⁵ and plated on both MRS and MRSE. Colonies were counted as in the above. The results are presented in Table 5. Numbers in Table 5 refer to 10⁶ CFU per ml.

Table 5

Comparison between wild-type and transformed
L. plantarum 80 as to growth rate

	MRS	MRSE	Ratio MRS/ MRSE
LPGA 6.3	1078	277	1/4
LPGA 6.6	1700	280	1/6
LPGA 3.9	1480	110	1/13

Table 5 shows that LpGA 3.9, 6.3 and 6.6 have growth rates comparable to L. plantarum 80 as was expected since strain LpG14 has the same growth rate as wild type L. plantarum 80. Again the engineered strains perform very well.

26.3. 220 g of non-sterile fresh grass was inoculated with respectively Lp80, LpG14, LpGA 6.6. In each case 10^{-6} bacteria per gram of grass were added. After respectively 1, 4 and 7 days two samples of 25 g of grass were suspended in 225 ml in MRS, serially diluted and plated on both Rogosa medium (R) (Oxoid Manual, 1982) and Rogosa supplemented with 10 µg/ml erythromycin (RE). The number of colonies were counted on both plates and converted to the number of bacteria per gram of grass. The results are presented in Table 6. Numbers in Table 6 are in 10^6 bacteria per gram of grass.

The figures for days 4 and 8 are the average of two samples.

Table 6

No inoculum			L. plantarum 80		
day	R	RE	day	R	RE
0	0.0006	-	0	(1)	-
1	10	-	1	180	-
4	89	-	4	380	-
8	130	-	8	165	-
LpG14			LpGA 6.6		
day	R	RE	day	R	RE
0	(1)	-	0	0	(1)
1	76	56	1	110	100
4	355	266	4	280	140
8	160	100	8	200	107

It is clear from Table 6 that the engineered strains constitute a considerable fraction (more than 50%) of the total population of lactobacilli, and that inoculation with transformed strains does not alter the proliferation profile of lactic acid bacteria. This proves that the Lp80 engineered to contain foreign genes as a chromosomal insert remains usable as a silage starter organism.

It will readily be appreciated that the invention thus provides a breakthrough in the art of genetically engineered, lactic acid bacteria useful as inocula in silage and as probiotics. It also goes without saying that this invention is not limited to the above examples. In this regard, other lactic acid bacteria, transformed in accordance with this invention, can be used as inocula in silage and as probiotics.

Also, DNA sequences other than those shown in Figs. 2 and 3 for the novel amylase gene and plasmid, respectively, of this invention can be used for transforming lactic acid bacteria. In this regard, such DNA sequences can be modified: 1) by replacing some codons with others that code either for the same amino acids or for other amino acids; and/or 2) by deleting or adding some codons; provided that such modifications do not substantially alter the properties of such DNA sequences. Furthermore, other DNA recombinants containing the aforementioned DNA sequences in association with other foreign DNA, particularly the DNA of vectors suitable for transforming microorganisms other than lactic acid bacteria, such as E. coli, B. subtilis and even eucaryotic organisms (e.g., yeasts), are encompassed by this invention.

Furthermore, other exogenous DNAs encoding enzymes can be used for transforming lactic acid bacteria in accordance with this invention. In this regard, exogenous DNAs encoding other proteins, which promote the degradation of polysaccharides and/or oligosaccharides to fermentable carbohydrates, can be used.

There follows a list of references which have been referred to above in describing the invention and its examples with regard to known methods for achieving some of the process steps referred to herein and with regard to general knowledge which has formed the basis for this invention.

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Claims

1. An inoculum for silage characterized by lactic acid bacteria, preferably a *Lactococcus*, *Enterococcus*, *Lactobacillus*, *Leuconostoc* or *Pediococcus*, particularly *Lactobacillus plantarum* and *E. faecalis*, transformed with an exogenous DNA which codes for an enzyme that breaks down an oligosaccharide and/or a polysaccharide into a fermentable carbohydrate.

2. The inoculum of claim 1, in which the exogenous DNA codes for an amylase, pectinase, cellulase or hemicellulase, preferably α -amylase, β -amylase, glucomylase, isoamylase, pullulanase, endoglucanase, exoglucanase, beta-glucosidase, pectic lyase, pectic glycosidase, pectic methyl esterase, xylanase, mannanase, arabinanase, inulinase, levanase or fructanase, especially an α -amylase encoded by the DNA sequence of Fig. 2, particularly from nucleotide 184 to nucleotide 2131.

3. The inoculum of anyone of claims 1 and 2, in which the lactic acid bacteria is transformed with two or more of the exogenous DNAs, at least one of which is preferably under the control of a promoter having a DNA sequence as shown in Fig. 7a.

4. The inoculum of anyone of claim 1-3, in which the lactic acid bacteria is transformed with at least one, preferably at least two, of the exogenous DNAs that code for a cellulase and with at least one of the exogenous DNAs that codes for an enzyme that breaks down a polysaccharide and/or an oligosaccharide but is not a cellulase.

5. A probiotic characterized by the transformed lactic acid bacteria of anyone of claims 1-4; provided the bacteria are not *Streptococcus* unless the bacteria are transformed with at least two of the exogenous DNAs.

6. DNA which codes for an amylase, characterized by the DNA sequence shown in Fig. 2, particularly from nucleotide 184 to nucleotide 2131.

7. A plasmid DNA containing the DNA sequence shown in Fig. 3a.

8. An ensilage method characterized by: the use of the inoculum of anyone of claims 1 to 4 on a crop.

9. A method for establishing and maintaining optimal intestinal flora in humans or animals characterized by feeding the humans or animals the probiotic of claim 5.

10. An amylase having the amino acid sequence shown in Fig. 2, particularly from nucleotide 184 to nucleotide 2131.

11. The transformed lactic acid bacteria of anyone of claims 1-4; provided the bacteria are not *Streptococcus* unless the bacteria are transformed with at least two of the exogenous DNAs.

12. A method for transforming lactic acid bacteria with an exogenous DNA characterized by electroporation of the exogenous DNA, preferably carried out in a low ionic strength buffer, with rapidly growing exponential phase cells concentrated to 10^{10} to 10^{11} CFU/ml.

13. DNA which contains the ORF 2, the nicking site, or both of the 1512 basepair Aval-SacI fragment of the plasmid of claim 7.

14. DNA which contains the ORF 1, the RS_A site, or both of the 1819 basepair Aval-SacI fragment of the plasmid of claim 7.

15. A method for detecting a microorganism, particularly lactic acid bacteria, transformed with two or more exogenous DNAs, one of which codes for an enzyme, particularly an amylase, characterized by detecting the enzyme produced by the microorganism.

1 KB.

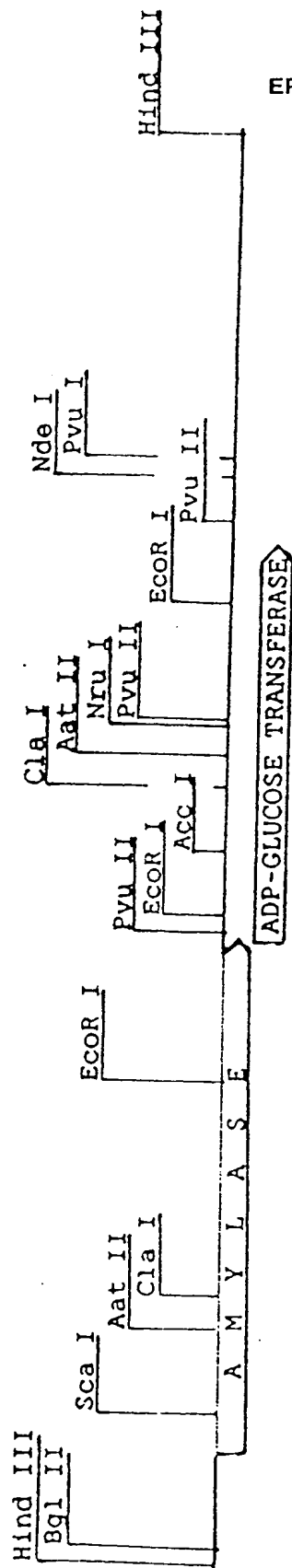


fig. 1

fig. 2

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1
 AAA TAT AGA AGT AGA ATG AAG CGT TTA CAA TGA ATA TTC GTC AAA ATT CGA
 52
 TAT TTT ACA CCG AAA TGT GAA GAA AAA CTA ACA TTT TTA AGA AAA AAA CGA
 103
 ATG TTT TTT ATA AAT TAA CAA GGA TAT TGC AGT TTT TCG CAG AAA TAG TTA
 154
 AAG TAT AAA AAA TTC TGT CAT CTA AGT GAG GTG AGC ATA TTG AAT GTA ATA
 205
 ASN CYS GLU GLU VAL LYS ARG ASP GLU PHE HIS THR GLU LYS TYR TYR GLU
 AAT TGT GAA GAA GTG AAA CGA GAT GAG TTT CAT ACA GAA AAG TAC TAT GAA
 256
 SER TYR ASN ILE PHE GLY ALA HIS VAL VAL THR GLU ASP GLU ILE GLN GLY
 AGT TAT AAC ATC TTT GGC GCG CAT GTT GTG ACG GAA GAT GAG ATT CAA GCG
 307
 VAL ARG PHE THR VAL TRP ALA PRO HIS ALA LYS ALA MET SER VAL VAL GLY
 GTA CGA TTT ACA GTG TGG GCT CCT CAT GCG AAA GCA ATG AGT GTT GTT GGG
 358
 ASP PHE ASN GLU TRP ASP TYR GLU GLN HIS LYS MET LEU GLN VAL THR GLU
 GAT TTT AAT GAG TGG GAT TAT GAG CAA CAT AAG ATG CTA CAA GTG ACA GAA
 409
 GLU GLY ILE TRP SER LEU PHE ILE PRO HIS ILE GLU GLU GLY GLU ILE TYR
 GAA GGC ATT TGG TCC TTA TTT ATA CCG CAT ATT GAA GAA GGA GAA ATA TAT
 460
 LYS TYR ALA ILE GLU THR LEU ALA GLY ASP VAL ILE LEU LYS ALA ASP PRO
 AAA TAT GCG ATT GAA ACG TTG GCT GGT GAC GTC ATT TTA AAG GCA GAT CCG
 511
 TYR ALA VAL TYR ALA GLU VAL ARG PRO ASN THR ALA SER VAL VAL PHE ASP
 TAT GCT GTA TAT GCA GAA GTA AGA CCG AAT ACG GCA TCT GTA GTT TTT GAT
 562
 ILE LYS GLY TYR GLU TRP ASN ASP LYS ASN TRP ASN ARG LYS LYS LYS LYS
 ATA AAA GGA TAT GAA TGG AAT GAT AAA AAC TGG AAT CGT AAG AAA AAG AAA
 613
 LYS SER ILE TYR LYS GLU ALA MET THR VAL TYR GLU LEU HIS PHE GLY SER
 AAA TCG ATT TAT AAA GAA GCG ATG ACA GTT TAT GAA TTA CAT TTT GGT TCT
 664
 TRP LYS LYS LYS GLU ASP GLY THR LEU TYR SER TYR ARG GLU MET VAL GLU
 TGG AAA AAG AAA GAA GAT GGA ACG CTG TAC TCT TAC AGG GAA ATG GTT GAA
 715
 GLU LEU ILE PRO TYR VAL VAL GLU HIS GLN PHE THR HIS ILE GLU ILE MET
 GAG CTC ATC CCG TAT GTG GTG GAA CAT CAA TTT ACA CAT ATT GAA ATT ATG
 766
 PRO LEU VAL GLU HIS PRO TYR ASP ARG SER TRP GLY TYR GLN GLY THR GLY
 CCG CTT GTT GAG CAT CCA TAT GAT CGT TCT TGG GGA TAC CAA GGA ACG GGA
 817
 TYR TYR ALA ALA THR SER ARG PHE GLY THR PRO HIS ASP LEU MET HIS PHE
 TAT TAT GCA GCG ACG AGT AGA TTT GGT ACG CCG CAT GAT TTA ATG CAT TTT
 868
 VAL ASP GLU CYS HIS LYS TYR GLY ILE GLY VAL ILE LEU ASP TRP VAL PRO
 GTC GAC GAA TGT CAT AAA TAT GGA ATC GGT GTC ATT TTA GAT TGG GTG CCG
 919
 GLY HIS PHE CYS LYS ASP ALA HIS GLY LEU TYR LEU PHE ASP GLY THR PRO
 GGG CAT TTT TGT AAA GAT GCT CAC GGT TTA TAT TTG TTT GAT GGT ACA CCG
 970
 THR TYR GLU TYR LYS ASP LYS ASP VAL GLN GLU ASN PRO VAL TRP GLY THR
 ACC TAT GAA TAT AAA GAT AAA GAT GTA CAA GAA AAT CCA GTA TGG GGA ACT
 1021
 VAL ASN PHE ASP LEU GLY LYS ARG GLU VAL ARG ASN PHE LEU ILE SER ASN
 GTC AAT TTT GAT TTA GGA AAG AGA GAG GTA CGT AAT TTC TTA ATT TCA AAT
 1072
 ALA LEU PHE TRP MET ARG TYR PHE HIS ILE ASP GLY PHE ARG VAL ASP ALA
 GCG TTA TTT TGG ATG AGA TAT TTC CAT ATT GAT GGT TTC AGA GTT GAT GCA
 1123
 VAL ALA ASN MET LEU TYR TRP ASN LYS GLU GLY GLN GLU GLN SER ASN GLU
 GTT GCG AAC ATG TTG TAC TGG AAT AAA GAA GGA CAA GAG CAA AGT AAT GAG
 1174
 HIS ALA VAL SER PHE LEU ARG GLU LEU ASN GLU ALA VAL PHE ALA GLU ASP
 CAT GCT GTT TCA TTT TTA AGA GAG TTA AAT GAA GCA GTG TTT GCA GAG GAT

1225
 GLU ASP PHE LEU MET THR ALA GLU ASP SER THR ALA TRP PRO LEU VAL THR
 GAA GAT TTT CTT ATG ACG GCA GAA GAT TCA ACA GCT TGG CCA CTT GTA ACA
 1276
 THR PRO THR TYR GLU GLY GLY LEU GLY PHE ASN TYR LYS TRP ASN MET GLY
 ACT CCA ACG TAT GAA GGT GGG CTT GGA TTC AAT TAC AAA TGG AAC ATG GGC
 1327
 TRP MET ASN ASP VAL LEU LYS TYR MET GLU CYS ALA PRO GLU TYR ARG LYS
 TGG ATG AAT GAC GTG CTG AAA TAT ATG GAG TGT GCG CCT GAA TAT AGG AAA
 1378
 HIS ILE HIS GLU LYS MET THR PHE SER LEU LEU TYR ALA TYR SER GLU ASN
 CAC ATT CAT GAG AAA ATG ACG TTT TCT TTA CTA TAT GCG TAC TCT GAA AAC
 1429
 PHE ILE LEU PRO LEU SER HIS ASP GLU VAL VAL HIS GLY LYS LYS SER LEU
 TTC ATA TTA CCG CTT TCT CAT GAT GAA GTC GTT CAT GCG AAA AAG TCG TTA
 1480
 LEU ASN LYS MET PRO GLY ASP TYR TRP ASP LYS PHE ALA GLN LEU ARG LEU
 TTA AAT AAA ATG CCA GGT GAT TAC TGG GAT AAG TTT GCT CAA CTT CGT TTA
 1531
 LEU TYR GLY TYR PHE PHE THR HIS PRO GLY LYS LYS LEU LEU PHE MET GLY
 TTA TAT GGA TAT TTC TTT ACT CAC CCA GGA AAG AAG TTA CTT TTC ATG GGA
 1582
 GLY GLU PHE GLY GLN PHE ASP GLU TRP LYS ASP LEU GLU ASP LEU ASP TRP
 CGA GAA TTC GGA CAG TTT GAT GAG TGG AAA GAC CTT GAA GAT TTA GAT TGG
 1633
 ASN LEU HIS ASP PHE GLU MET HIS ARG TYR MET HIS ASP TYR PHE ASN GLU
 AAT TTA CAT GAT TTT GAA ATG CAT CGT TAT ATG CAT GAT TAC TTT AAC GAG
 1684
 LEU ILE ALA LEU TYR LYS ARG SER LYS PRO LEU TRP GLN LEU ASP HIS SER
 CTC ATA GCA TTG TAT AAG CGC TCA AAA CCA CTT TGG CAA CTT GAC CAT TCA
 1735
 PRO GLU GLY PHE GLN TRP ILE ASP ALA ASN ASN ASN GLU GLN SER ILE PHE
 CCT GAA GGT TTT CAG TGG ATT GAT GCT AAT AAT AAT GAG CAA AGT ATT TTC
 1786
 SER PHE ILE ARG GLN GLY ASP LYS GLN GLU ASP ALA LEU VAL VAL VAL CYS
 TCT TTT ATC CGC CAA GCG GAT AAA CAA GAA GAT GCG TTA GTT GTC GTA TGT
 1837
 ASN PHE THR LYS ALA THR TYR GLU ASN TYR LYS VAL GLY VAL PRO ASP PHE
 AAT TTT ACG AAA GCT ACA TAT GAA AAC TAT AAA GTA GGT GTA CCA GAT TTC
 1888
 GLU TYR TYR ASN GLU ILE LEU ASN SER ASP ALA GLU GLN TYR GLY GLY SER
 GAG TAT TAT AAC GAG ATT TTA AAC AGT GAT GCT GAG CAA TAT GCG GGT TCG
 1939
 GLY GLN VAL ASN LYS LYS ARG LEU LYS THR ILE LEU GLU PRO TYR HIS ASN
 GGG CAA GTG AAT AAG AAA CGT CTT AAG ACG ATT CTA GAA CCG TAC CAT AAT
 1990
 GLN ALA ALA HIS VAL GLU ILE THR ILE PRO PRO PHE GLY VAL SER ILE LEU
 CAA GCA GCG CAT GTA GAG ATT ACA ATT CCA CCA TTT GGC GTA TCC ATA TTA
 2041
 ARG PRO VAL LYS THR ARG LYS GLY SER LYS LYS GLN ASP GLY SER LYS THR
 CGA CCA GTG AAG ACG AGA AAG GGG AGC AAA AAA CAA GAT GGC TCA AAA ACA
 2092
 LYS VAL ARG SER ASN VAL THR SER ARG GLY LYS ARG
 AAA GTG CGT AGC AAT GTT ACT AGC AGG GGG AAA AGG TAG TCG TTT AAG TGC
 2143
 ATT AAC AAA AAA TTT AGC CAA GCC AGC TGT TCC ATT TGG TGG TAA ATA TCG
 2194
 CAT TAT TGA TTT TAC GCT AAG TAA CTG TGC GAA TTC TGG TAT TGA AAC GGT
 2245
 TGG GAT TTT AAC GCA ATA TCA ACC ACT TGA ACT TCA TAA TTA TAT CGG AAT
 2296
 CGG AAA TGC GTG GGA TTT AGA CCG AGT AAA CCG TGG GGT CAC AGT ATT ACC
 2347
 TCC TTA TGC GGA GTC TTC AGG GGT TAA GTG GTA TAC AGG TAC GGC AAG TGC
 2398
 CAT TTA TCA AAA TTT AAA CTA TTT AAG TCA GTA TGA ACC GGA GTA TGT TCT
 2449
 TAT TTT ATC AGG CGA CCA TAT TTA TAA AAT

Figure 3a:

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66
K L C L C L L F F G L Y F F Q F F P H S F V
AAGCTTTGCTTGTGCTCTTGTCTTTTCGGTCTCTATTTTTTCAATTCTTCCCGCATAGCTTTGTA
L K A Q T E Q K E T E I K K L E E R M A K Y
F G Y * STOP ORF 4 132
TTCGGGTACTGATAAATTCTTACGCTCTTTATTTTTGTTACCACGTTGAACATCAAAGCCATTTC
E P V S L N K R E K N K N G R Q V D F G N E
198
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K L Y Q P L E E Q I R Q L A E R N F I R K A
264
TGAGAGCTTTTTATCATCATCAAAGGGCACAATGCCCATATGCATGTGAGGGGTCTTCTCGTCCAT
S L K K D D D F P V I G M H M H P T K E D M
330
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H V V A Y R I N E D G Y N D A F Y Q K A T E
396
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F Y K R T E A E D L Q E F F D K D S T I I W
462
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E N V L V A D K R V A R K S A K N E N I Y A
528
TTCAATATCCGTTTTAAAGTTATCAGTGCGCCCAGCCACTAAATCATAATTCAGGTGAGAGTGGA
E I D T K F N D T R G A V L D Y N L H S H S
594
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V D I D E N S H N T T K R Q D H N G I G V L
660
ATTATCAGCTTTTAATTTTCGTCATTCTAGCCACTGCAAAACTCATTGTATGCACCTCATTTTCGGAG
N D A K L K T M R A V A F S M START ORF 1 SD
726
TTACCTTCCAAGTAAAGTATAACCCACTATACTTTATGTTATAAAGTGTGAGCTCTTTCAGAGGGA
-10 -35
792
CGCCTTGCCAGCGGGACAGACCCACCGCTTATTTTTCTGACGAAAAATTCCGCTCTCGTTATCGA
858
AAATTTTGGCTGTTTTTTTACGCCAAAATAATCTCAACTCACGTTTGCTTTTTCAAGCAAAAAGA
924
ACGGATACTAGCTCCGTTCTTTTCCCTTTACCAGATAAAATAATTTTGCTTATTATAATCCCATT
STOP ORF 2 * W I F Y N Q K N Y D W K

TCGCAACGACCTCGCGAACCACCTCATCGATTTTACTTTATCATCATCGACATTAATTAAGTGAG 990
 A V V E R V V E D I K V K D D D V N I L H A

CATCAACATCTTCAAGTTGTAAGTCTTGCGAATTTCTTTAAATAACCCACCATAACTAATTTGAC 1056
 D V D E L Q L Q K R I E K F L G G Y S I Q R

GTGACCCCGCCAATCCCTGTTCCAAATCCTCAACCACTTGCGAGTGCAGTTCCTTGGTCATTCGTCA 1122
 S G A L G Q E L D E V V Q L D R E Q D N T L

^e AAATATCTTTGGACTTAACTTGATATTTAGCGGTTTCTTGTGCACTCGCAATTAACGAGTTTAC 1188
 I f D K S K V Q Y f K A T E Q A S A I L S N K G

CTTTAGCCTTGTTGAGCGAACAGCCTCAACATTAACAATTGGCTGATAATCTAACTTCATAGCCC 1254
 K A K N S R V A E V N V I P Q Y D L K M A R

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 Q W L K S W E A Q N I Y N A S D K F Y T P K

TAACGAAAAGCAGCACGTGCATGTGCTGATGATAAGTCCCATTTTGTGATCGTAATTTCCGTTG 1386
 V F L L V H M H Q H Y T G N K N I T I E T S

AACGCACATAACCCAACAAGTTTTTGGCCGGTTTCTTGTATTGAAACAGCTTAGAAATTGCCCGTC 1452
 R V Y G L L N K A P K K Y Q F L K S I A R G

CCATTTTCTGACTTCCTGTTTCAAGTTTTCGCCAGATGCGTTTTCCGCCGTCAGCGTTAAAAACA 1 1518
 M K R V E Q K L N E G S A N E A T L T L F L

AAAAGCGACCCGTTTTTTCGTTGCTTATGAGCTTCGTCTAAACTTGCAATTAAGTATTGATTGTC 1584
 F R G T K R Q K H A E D L V Q M L Q N S Q G

^l CCATTGAGCGACGCCAATTGCAⁿCAATGGAC^lCAATCGACTCTTACAAAACCATGTCTGATAAAGCC 1650
 M S m R R W n N C L P C L R S K C F W T Q Y L R

GCAGTCGGCCCTCATCATCAGCAACAATCTCAATACTTCACCGCATTCTTTAACACGATTAGCTT 1716
 L R G E D D A V F m R L V E G C E K V R N A K

TTTTAAATTAAGTATTCTCAAATACTCCGCGTACTGTAAGTTTTCCAATTTACGTTACAGCCACG 1782
 K F N L I R L Y E A Y Q L N E L K R E R W P

GCCGTTCTTTTCTGTTACGACTAACATCTTTTAAATCTTTTTCGACATAAAAAATAGCTCCAATCC SD 1848
 R E K R N R S V D K L I K K S M START ORF 2

AGCAATGAATTTCTCACGTGGCTTGGAGCTAATCACAATTCGTAAAGTATAAAAAATAGACTTTAGAT 1914
 -10
 AGTCAACGTTGACAAATAACTCACTTGTCTAGTACTTATCTCACGTATATTATTGATTAGCTCAC 1980
 -35
 GTGTCCTGCTCGCCAAAGTTAGGATTTCCACGTGAGTTTTTTTATGCAGTTTTTTTAAATAATCTAA 2046
 q
 GACAACTGAGTCAATAAAAAGTTCAAAACCCTCAGTCAACTTTAATAAGCCAAATTATGCACTATAA 2112
 STOP ORF 3 * L
 ACGCCGGCCTGTCAACGCTTTACAGCAAAACCAAAAAAATGACGTTGTTTGTAGTAGTATCAAGAT 2178
 R R G T L A K C C F W F F S T T Q L L I L I
 AAGAAGAACTGGTCAAAATCGACCAGTTTCCAAGCCCTCGGGCGCTGACGCCCCGAACCCAGTCC 2244
 L L F Q D F D V L K W A R P R Q R G S G L G
 AAGCTGTACCAGCTTGACCCTATAATTGAGCGGGGTCCCCGCTCAAACCTCACTCCCACTCGCCGT 2310
 L Q V L K V R Y N L P T G R E F E S G S A T
 GTGGCAGGCGGAACAAGTGGTAAATCTACTTGTTCGGGCAAGCCGGTTAGTGTCTAGATTTCGAGTA 2376
 H C A S C T T F R S T G A L R N T D L N S Y
 ATGCTGTCAATTCCTTATCCTCCCACGCTCCGCTCGTCCGGTACCATTGACAGCGGCAGCATTAGC 2442
 H Q START ORF 3 SD -10
 CTCTCAACATTTTATTGAGTACGCTTACTTTTAGCAATAAAATATAATTGTACTAAGTATTTTAA 2508
 -35 x y
 GGAGGAATATACTCATAAAAATTATTAAATCAGTTACCTTTGCTATTGTTGGTACAACACTTTTAG 2574
 z
 CTACTAGTTTTCCCGCTATTCAAGCACACGCAAACGATCAAACAATAGAAAGTAATACAGTCGTTG 2640
 z
 AAAGTTTCGGTTGGCAATAAGGATTGGTTATATATGAAAAATAGCCTATGGATTTCGAAGTATACTG 2706
 ATCATCGCCTTATTATTTATTTTATGGGCAAGTAACCATTACAATTGGCCTTATCACAATATAACA 2772
 y
 GGATATGTCTTAATAGTATGTGGAGTATTAGTAGGACTTTTAGGTAATCAAATAGACAAAAAATAA 2838
 CAGCTAAAATAGCATATCTAGCAAGCCTTTTAACCGGTAAAGGAATCTAAAGGCTTGGACCATCTA 2904
 STOP ORF 1 * L S P G D L

297C
ATTCCTCATGGTGTCCCTCTGAACGCTCCTGTGAGCTTTCAGGGGCTTTTTTAGACGGTTCTTTTA
E E H H G E S R E Q S S E P A K K S P E K L

-35 3036
GTCCTGCACGCTCTAGCCACTTTTCAGGTAAATTAATACCTAGTTTTTTGTGTAGAAACTCATCAA
G A R E L W K E P L N I G L K K H L F E D V

-10 3102
CATTTTCGTATAATGCCTTGAAGCGTTCCTACTAGTTTTTGCAATCGCTCATTTTCTTTTCTCAGTT
N R I I G Q L T G V L K Q L R E N E K R L E

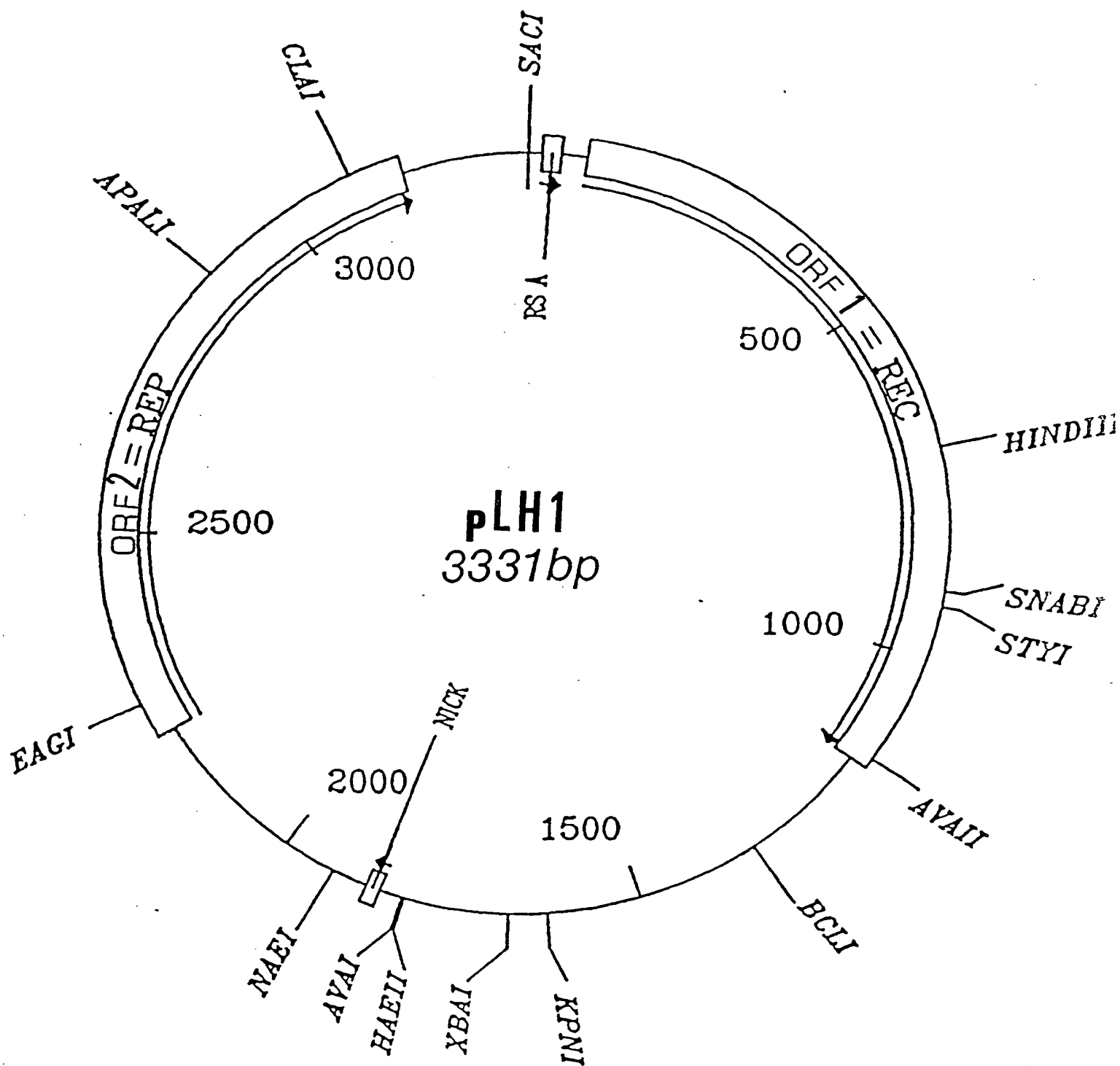
SD START ORF 4 M I F Y F Q M N 3168
CATAGTTGCGGCCCTTGGCTTCACTCAAATCATTACGTAAACTATGATTTTCTACTTCCAGATGAA
Y N R G K A E S L D N R L S H N E V E L H I

3234
F F K V I S V A S H R P L F Q I S E I R F I
TTTTTCAAAGTTATAAGCGTCGCTAGTCACCGCCCTTTGTTCAAATCAGCGAGATCAGATTTAT
K E F N Y A D S T V A R Q E L D A L D S K D

330C
N R D F I F I H Q G G F T S N F F G I P W F
CAACCGTGACTTTATTTTATTCATCAAGGTGGGTTTACTAGCAATTTTTTTTGGTATCCCGTGGTT
V T V K N K N M L T P K S A I K K T D R P K

3331
D F I K L F F S I C
TGATTTTCATCAAGCTGTTTTTTAGTATCTGC
I E D L Q K K T D A

Fig. 3a Cont. 3



REC110
RRC194
REC181
REC1

[illegible]

1444 3kLqQk.nvFv rQellWLDQFpFnmkqGfELkqERSDrKhIftakfkqtLeIdFleKnLavkKQEWtaysdkvkSdEvpakzhnks vevptGE kmfSGJgkeImktekptknvvisERdyknLv
 1445 3LSAKeqLGNKK dftQLDQFNEvYvnekGYEL ERCTSkvETzreHKamDQYKdVFNKQELqevKQELqkankqlqGgiEhm.....fStKP faye nertGL FSGreetgrkiltadeFER LQ
 1446 3LSAKEvGvNKKAL TaLDQFNEvHvKQYQGL ERQgSrQvTnaKHeqisQYkqkTEYKQBYeresqTdhikqkndklmqeyqkSLntLtkkfinvpyFqetekvGL FS keIQeagnvvisqkdfnefQ
 1449 kKLSAK.rifNreAlqriqeelpaylkengfadvORG nkKvER knlsbpyKamreelkKtEFG KQETqakladTtkqldeikprdtKkIaSkpTLmnknkv tvdKsdldladleqravtsdaynfekInIevenhsl
 1450 gFdsAK.vgnKkal---qe-t-lqdrfnehvkardyealk--ERg-sr-vte-kh---qYketeFhkqelq-kkdet-k--dk-ks--e---ks-ntl-kp--v-ye---k--gl-fs-r--e---nvviserd-n-lq

2777 taardndRIkqhvrNlMStDmaZEYxkXlSkqhqqvKEKysglvErfEnvndiNelieENkSlkSkisdIkrDvSllyestkefIkertdglkafKxnvfKGFvdkvdkdaqfQekhdlepkknefalthnrevKkErsRdgqmsl
2780 etIssAerIvdYENIKStDyytEnqeLkKtresLKEYvntwKEgYhEskeyNkLkrENdSL neqlnVS ekfgdstvTlyraaranFpQKGFnrkEkffndskfervQfmdvvQUnvQKvDRKrEkqRtdllem
2786 kqikaAqdIseDYeYIKSGralddkKefreKddLlnkaverfEnhadnfnqlYenaKpIkenIefalkllkIlkElervlgrnTfaervnkltepeKlIngIaglnldKXmnpelyseQeqqeQgKqNrDrgmhl
279 rndlseakgrnvelrKeneRlqlkvglvlgIirnvdeflhKklnglnpKwleragIKepskapesqersegHhEeldgpslIplpwkriaryailawIcfldyIkvlIlhlIlhlllycdkancngylpik

ff

RSAE	pE194(1-44)
RSAT	pT181(1-38)
RSAU	pUB110(1-38)
RSAL	pLAB1000(1-38)

RSA

- 35

[illegible]

ccgactTattaA-----aAaaaAGTatAGTGTGTTAGACTTAC

6
6

REP194	pC194(1-230)
REP1	pL1000(1-314)
REP110	pUB110(1-334)
REP14	pFTB14(1-339)
REP111	pX174(1-341)

1117 ynnsrKLlkrKKkvgsvikzrvskLElTynKKrdnphFHVlIaVnksYFDkryIIsQqEldlW
rdVtGiseltqvqvkirqnnnkelyEnAKY sgkKD
1126 MgraisKLfQKKpKaKNLlGYvrsTElTINK ngtKhQqMhVtIl fVRPTFKDSaNYINJaEWSkIwqzAMKLDY
qpivNVeaVrsnkaKgnS lIaSa qETAKqYvK sKDlITnDqEdLqvvvDLdEqdLa
1145 NaqGFRmmQYKdKnKVLvCPmRATeVlNhnKdSnQqFHVlcvjETFKntelVnDxqHl qWkKtAKMLDY
dpNVKvgmlrpknykSD IQSaldETAKpVWD tDfmtCdEeANLArIsDlEEqLh
1139 MneGFRklfQYKvKtSVLcfFRAlIiNkNheedtPhpHfHVtIlpVKnYF gKvYKpDaetSlwKzAKWLDYtpvdlrVrVKgrvkIdaeqIeSDvreammeQkAvLElIsKpVWddvVvrgsKVYtdnLnTcvfYlddLaLa
rlRgKVTVadvlaaqpvttnl
1138 rytqdaFs=sgwIwpvdaKpplkatsymavgfyaKvynkksdmdlaaKglgaKewnsSkEtLslIlpKklfrImrsnrfgmKmltmtnIsteclqltKlgydatpfnqIjKqKqKremlrlRgKVTVadvlaaqpvttnl
m=--gfKlfcgYkKv-knLkGfvrateItInkk-dtyhqbmbhVll-vkptyfkd-knyinqaew=-lwkrAmkldy-----I-nvK-vm-I-a-----sd-----Iqsa--eIdKypvkd--kdrItkdte-nl--v-vdie-gi-

```

220          sdyf INksks1
257 gsRqISYGGlfKEI rKqlqledvdahf INvDDdkvKlDEvzvrevvAkWdYnkqNYfiw
276 rKRLISYGGllKEIHKKLNvdDeEGDL InhDDD EKadEdgSfiliAmWnerKNYfIKE
281 arRLIgyGGllKEIHKeLNLgDaZgGGLvhlleEDD E vanGafevmNyWhpgIKNYlLK
282 kfmrasikImIvsnlgsfiasmtqkltlqsdIsdsksnyldkaGittacrlrkskwtaggk
--rlisyygllkeihhxxlnl-dte-gdl-In-ddd-ok-dog-fev-g-w----knyfik-

```

Diagram illustrating the DNA sequence of the B gene, showing the coding strand (top) and the template strand (bottom). The sequence is divided into five segments, each labeled with a number (1 to 5) and a protein name. A 'nick' is indicated by a downward arrow between the two strands at the position of the TCTTATCTTGCAT sequence.

Sequence 1: NICK194 (1-30)

Sequence 2: PUB110 (1-30)

Sequence 3: PFTB14 (1-30)

Sequence 4: PLAB1000 (1-30)

Sequence 5: IX174 (1-30)

The DNA sequence is shown in a double-stranded format. The top strand (coding strand) is 5' to 3' and contains the sequence: ATGAGG, TATAGA, TATAGC, CTACAC, and TATTTT. The bottom strand (template strand) is 3' to 5' and contains the sequence: TACCTT, ATCTTATCTTGCAT, TCTTATCTTGCAT, TCTTATCTTGCAT, and TCTTATCTTGCAT. A 'nick' is indicated by a downward arrow between the two strands at the position of the TCTTATCTTGCAT sequence.

Sequence 1: 1 TCCCTT ATCTTATCTTGCAT A

Sequence 2: 1 CTGCTTATCTTATCTTGCAT A

Sequence 3: 1 GGTCTTATCTTATCTTGCAT A

Sequence 4: 1 GTTCTTATCTTGCAT A

Sequence 5: 1 CTCTTATCTTGCAT A

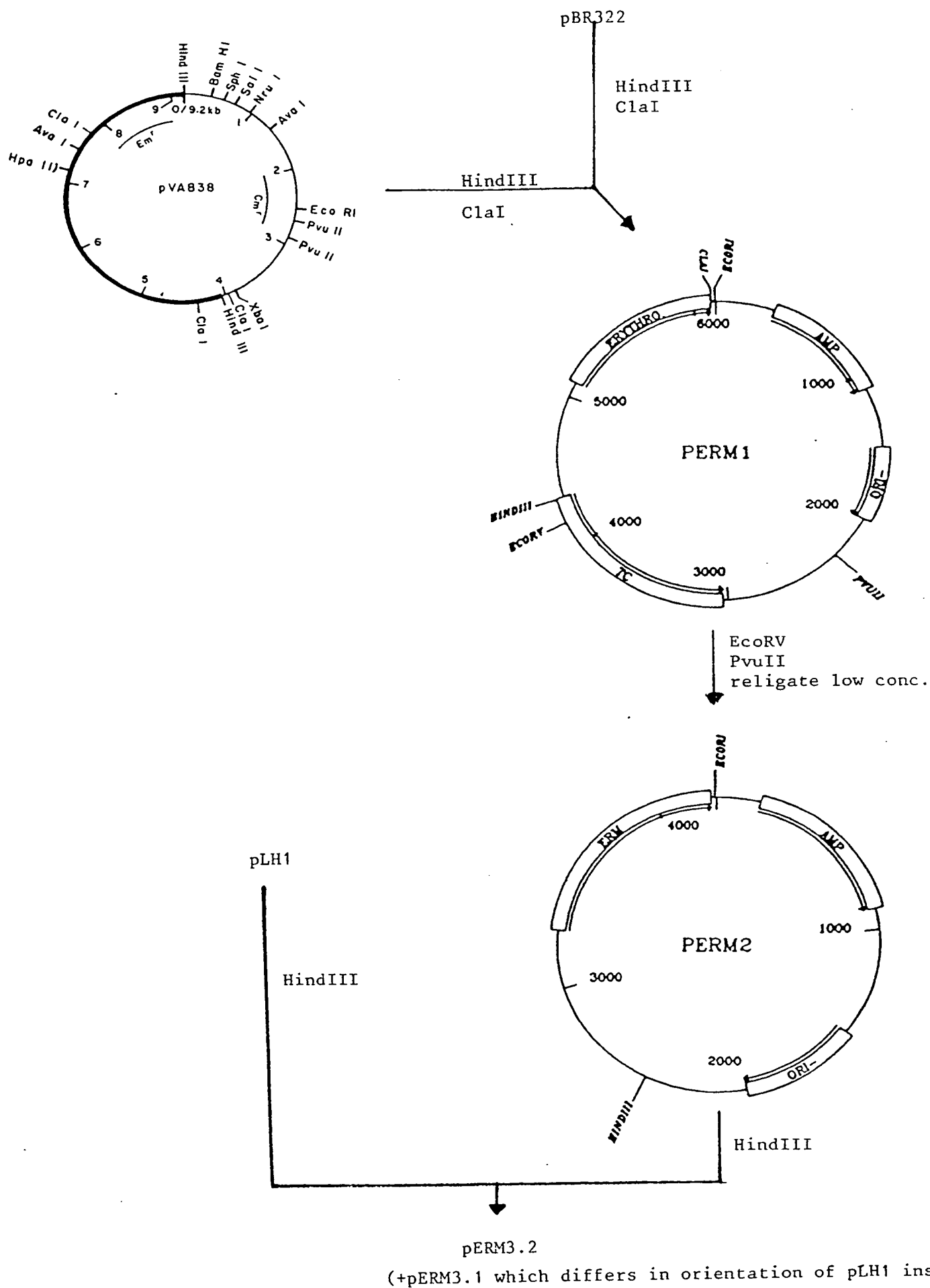
The sequence is flanked by the following sequences:

Left: --cgtagctctt

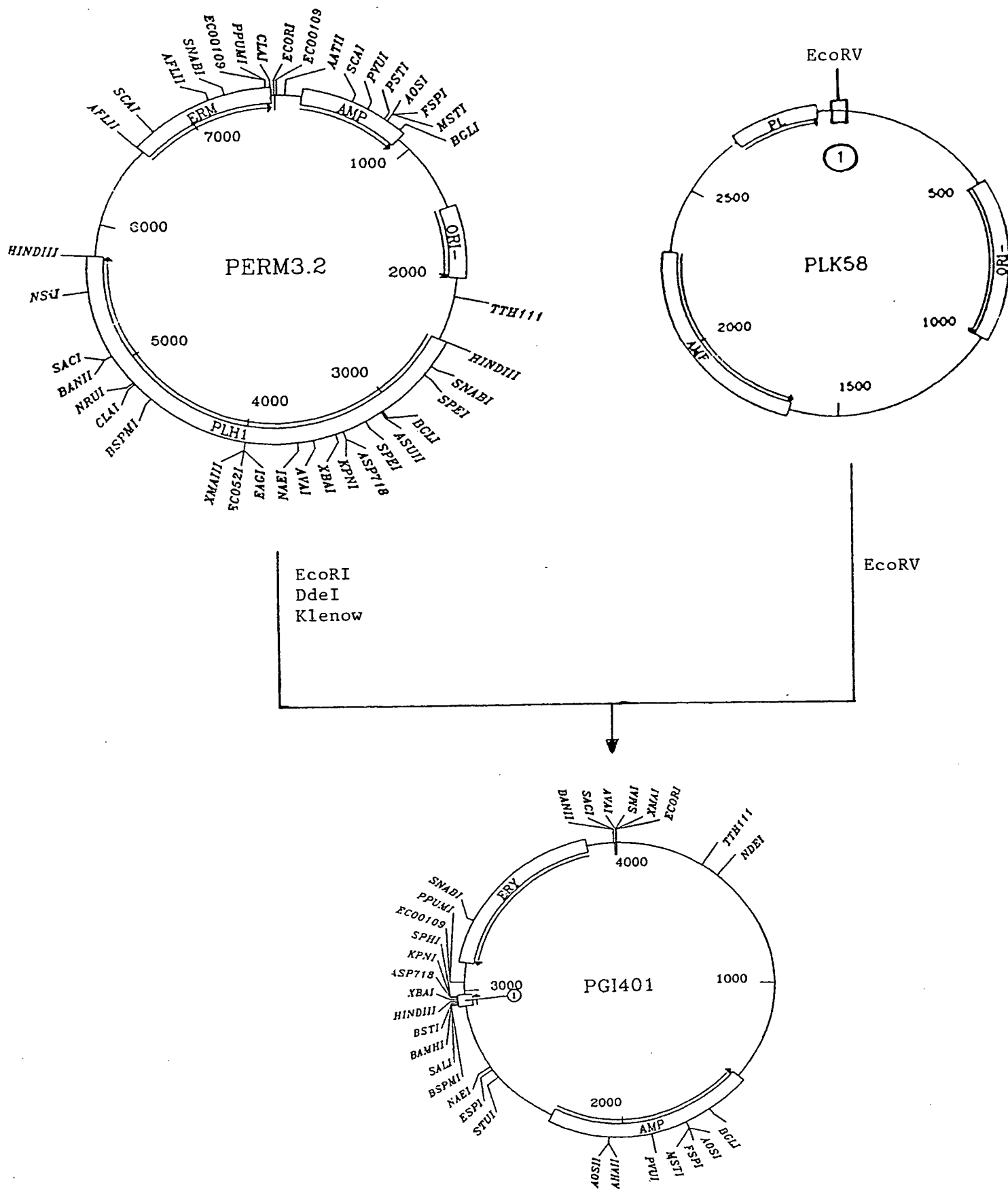
Right: --tatcaga--

Fig. 4

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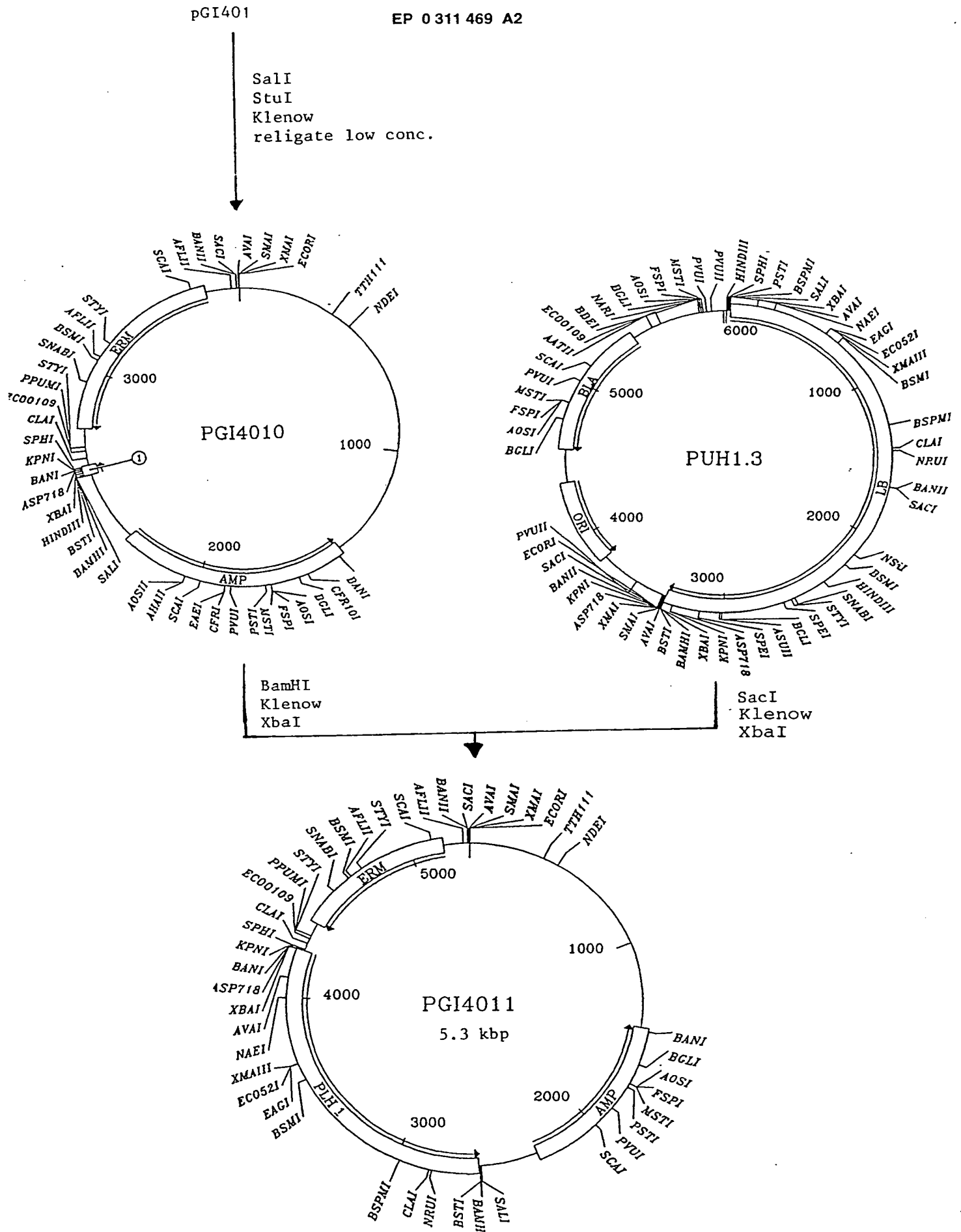


Fig. 5

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Bacillus stearothermophilus

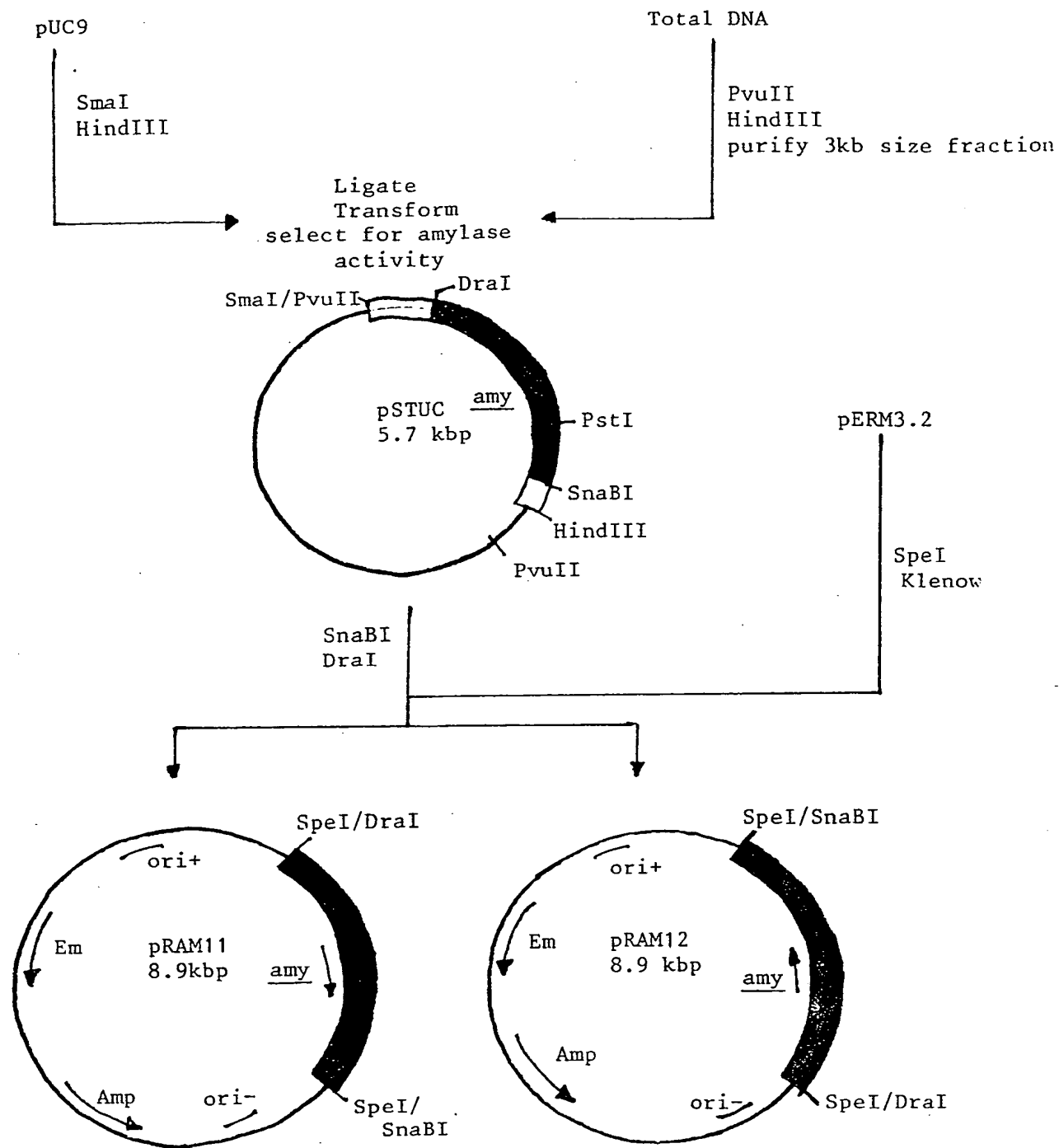


Fig. 6

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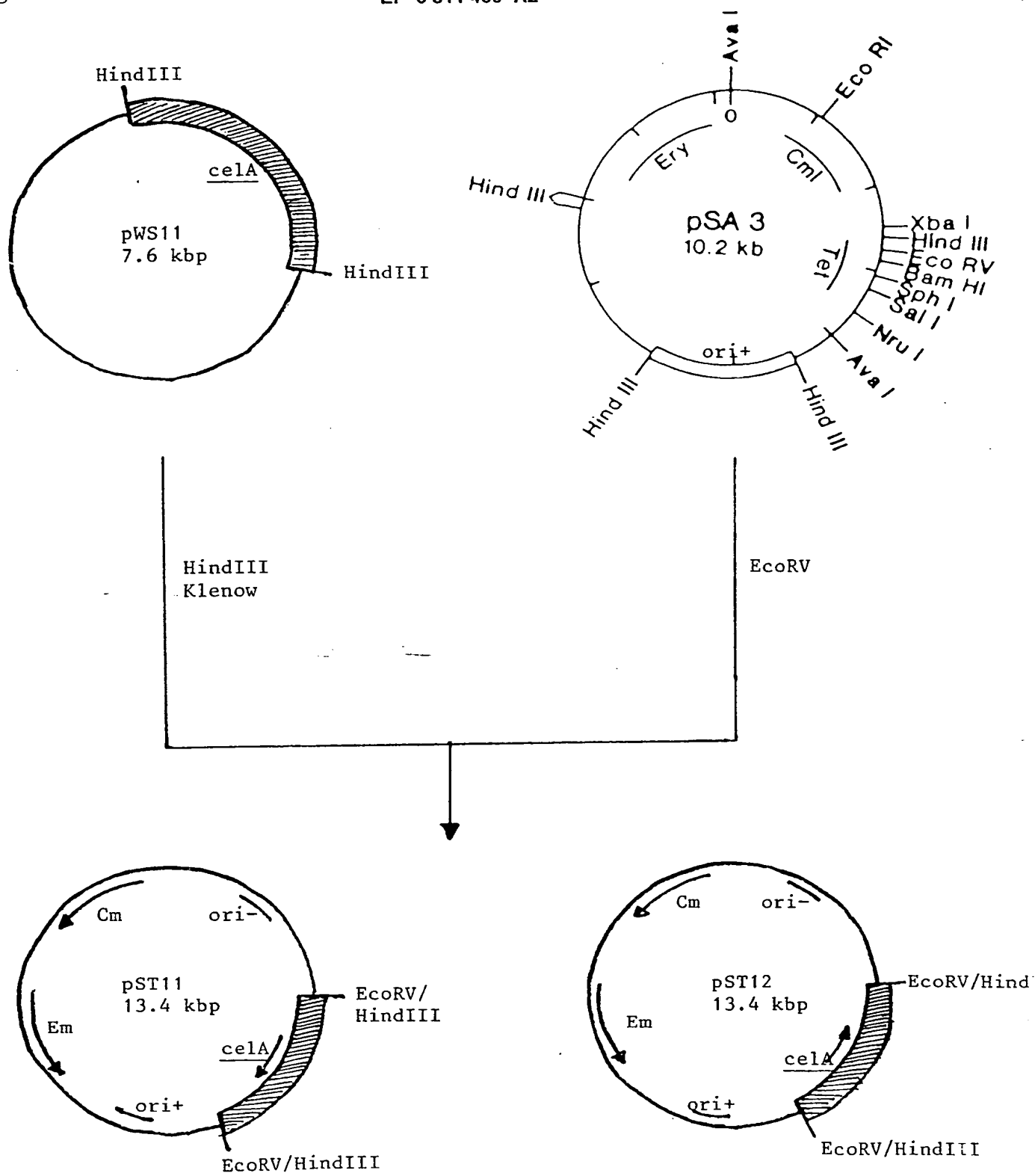
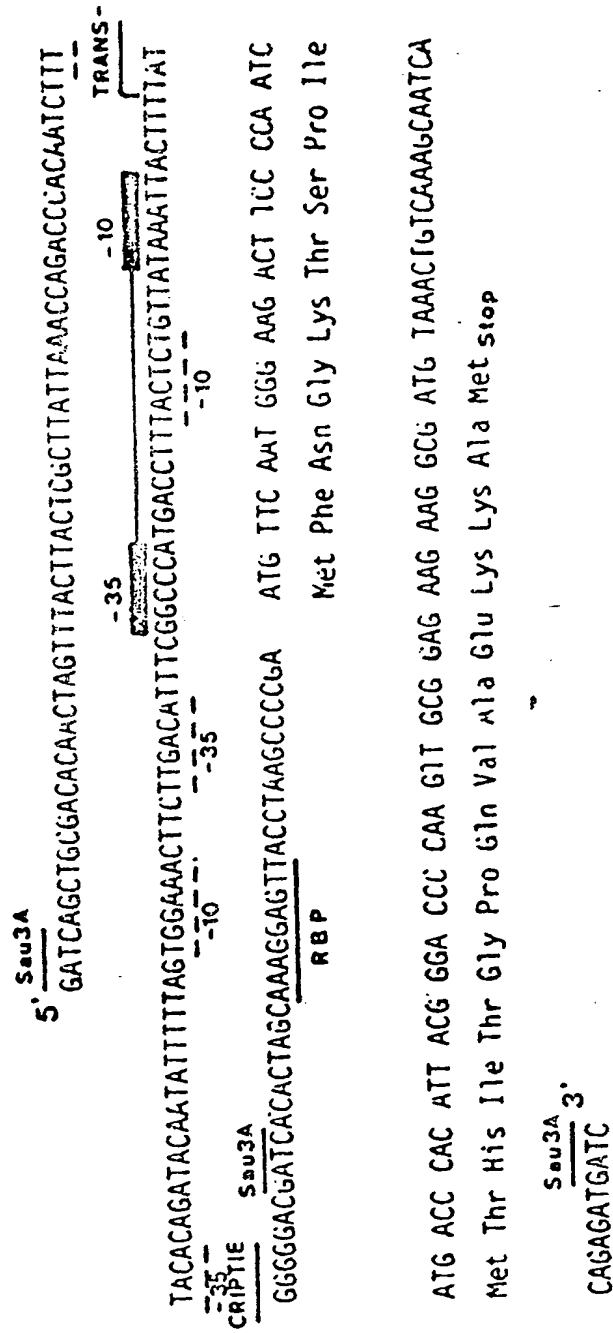


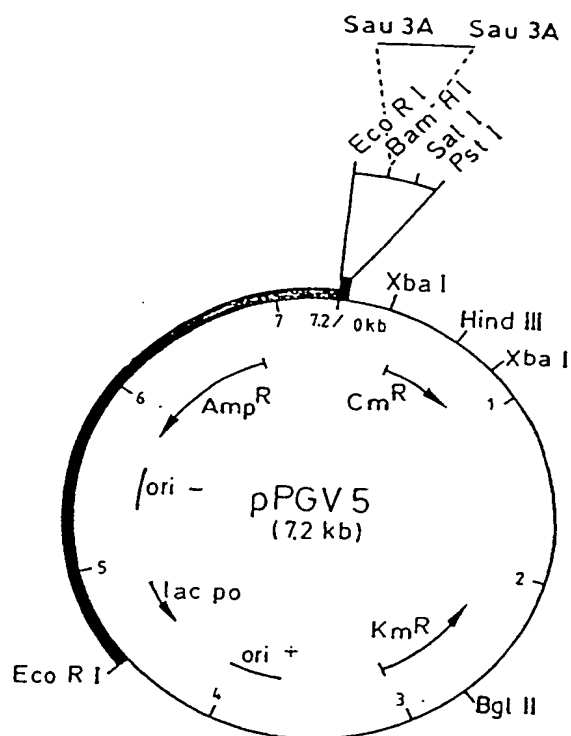
Fig. 7 a



Lactobacillus hilgardi 67

chromosomal DNA

Sau3AI



ligate
transform in *B.subtilis*
select for chloramphenicol
resistance

BamHI



e.g.

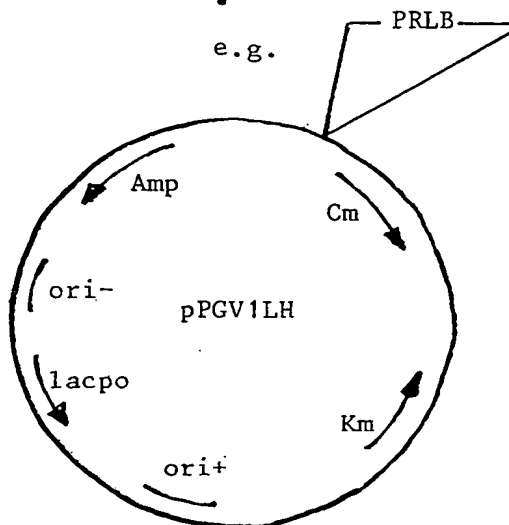


Fig. 7c

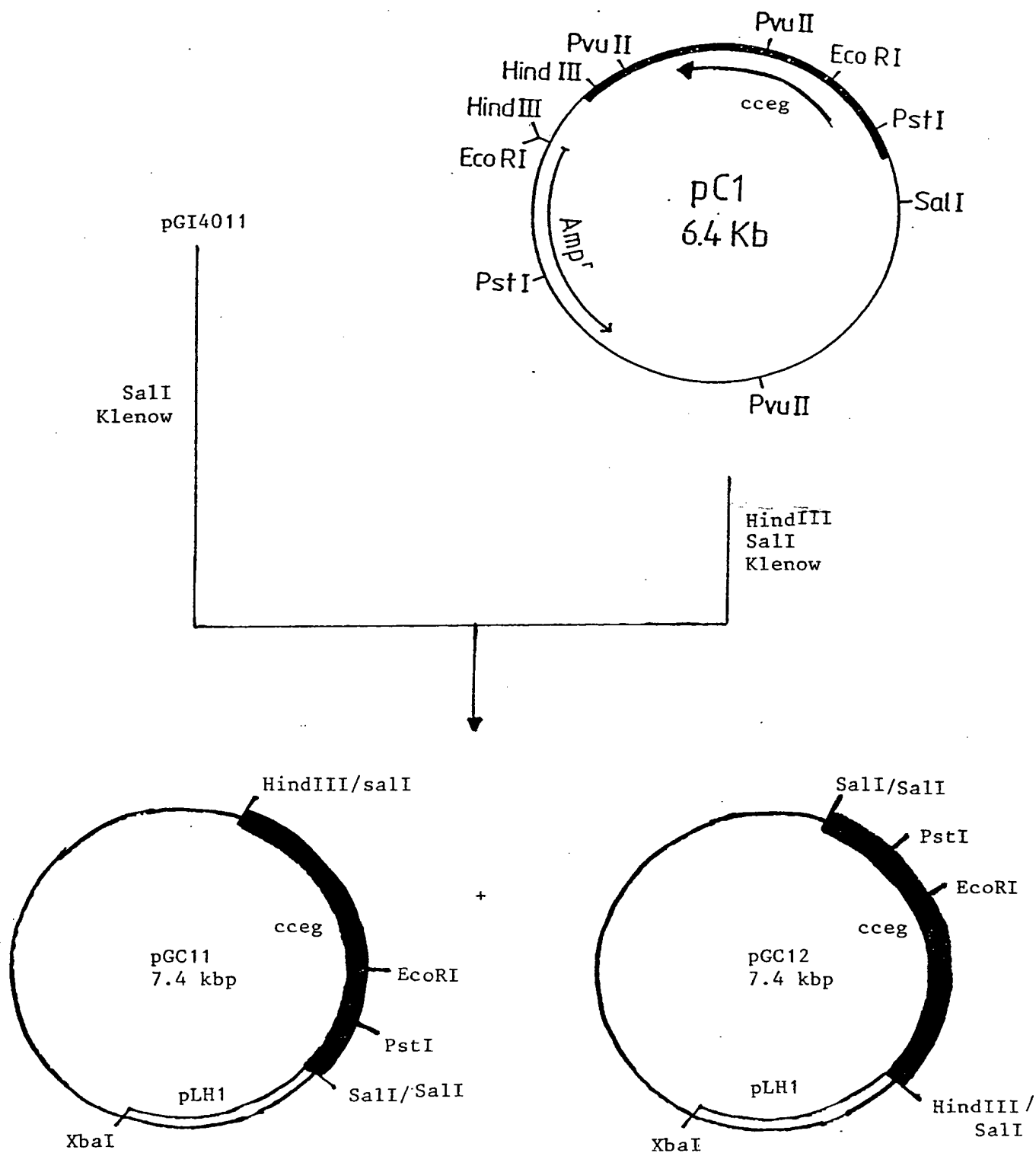


Fig. 8

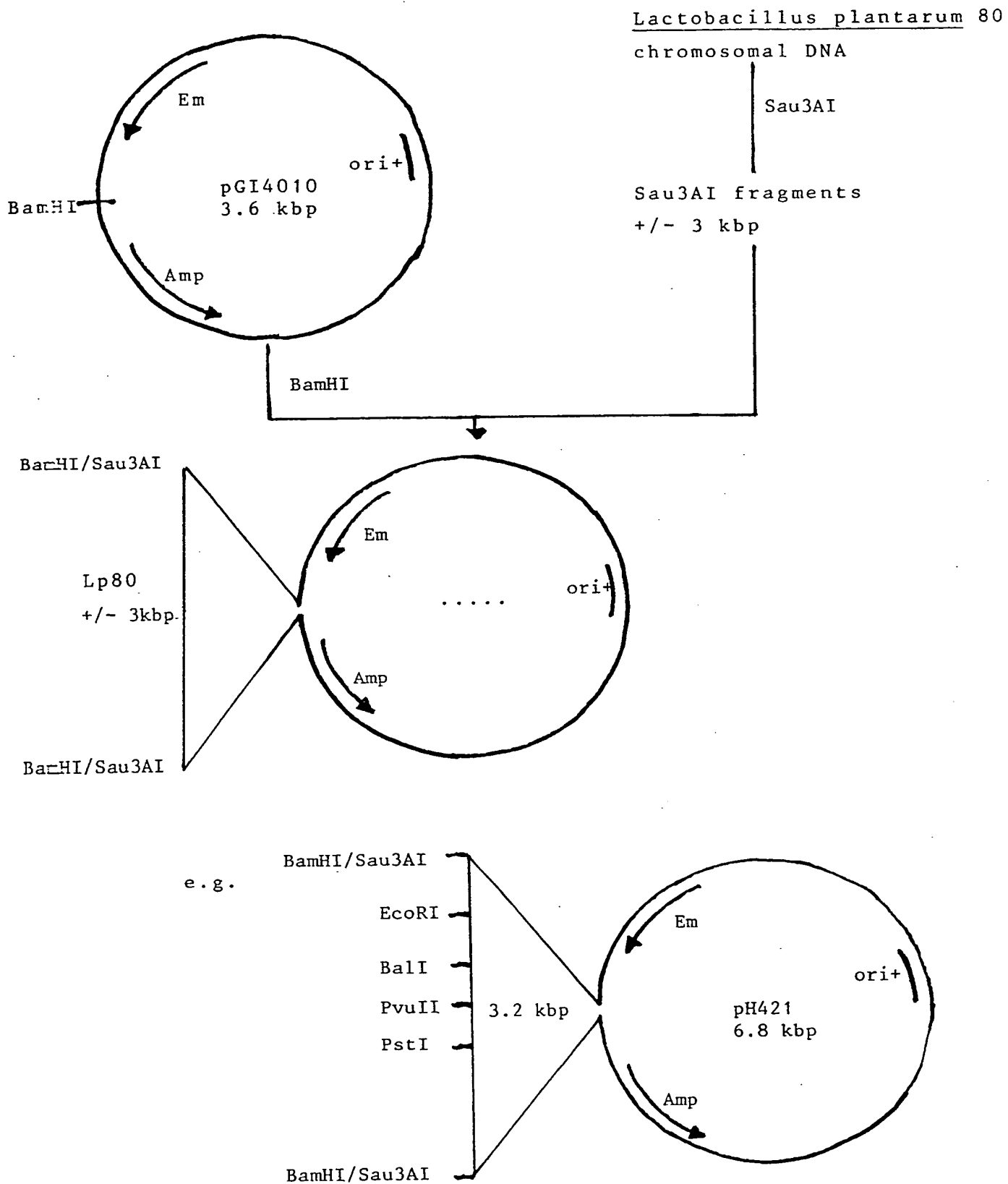


Fig. 9

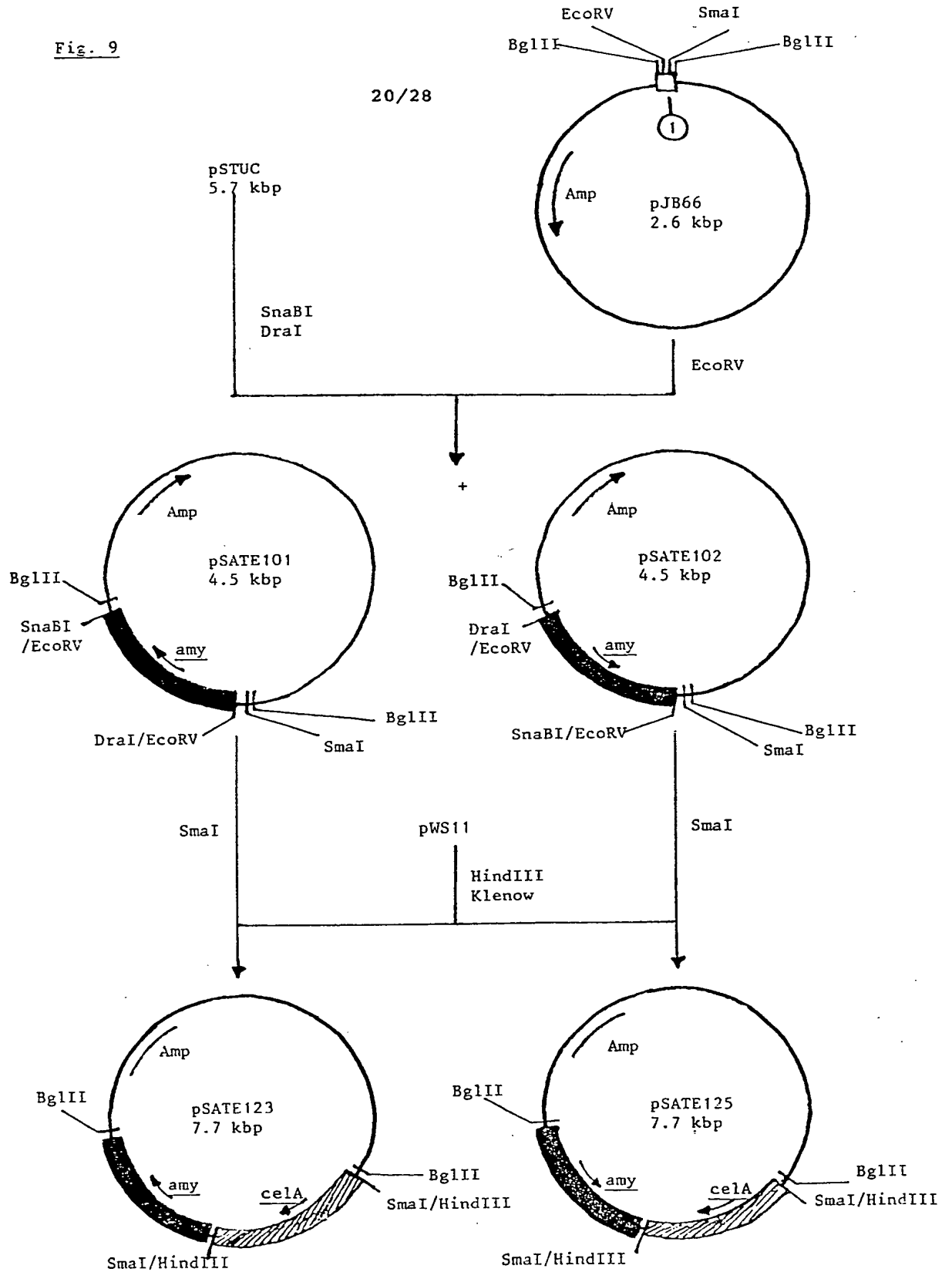


Fig. 9 continued

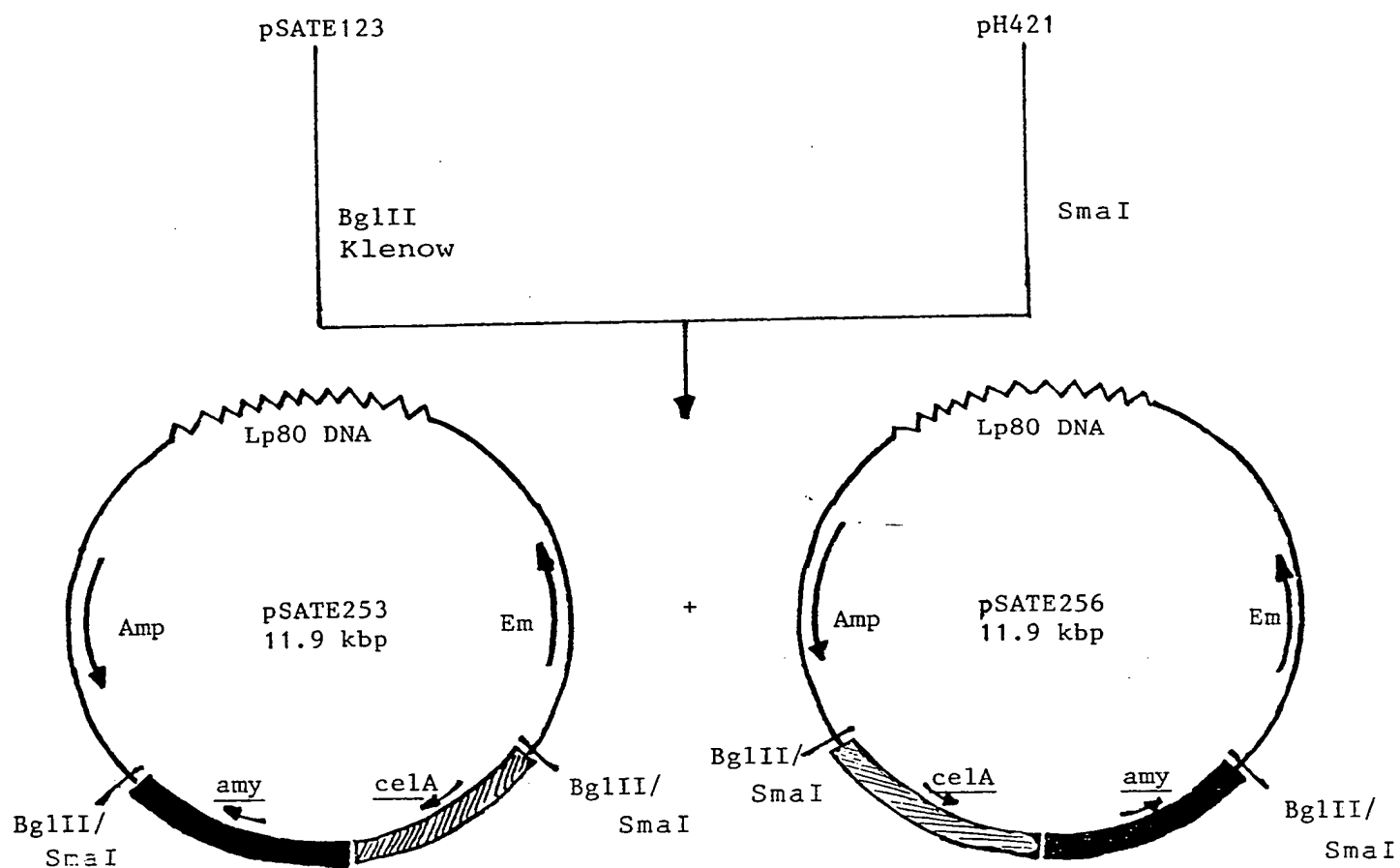


Fig. 10

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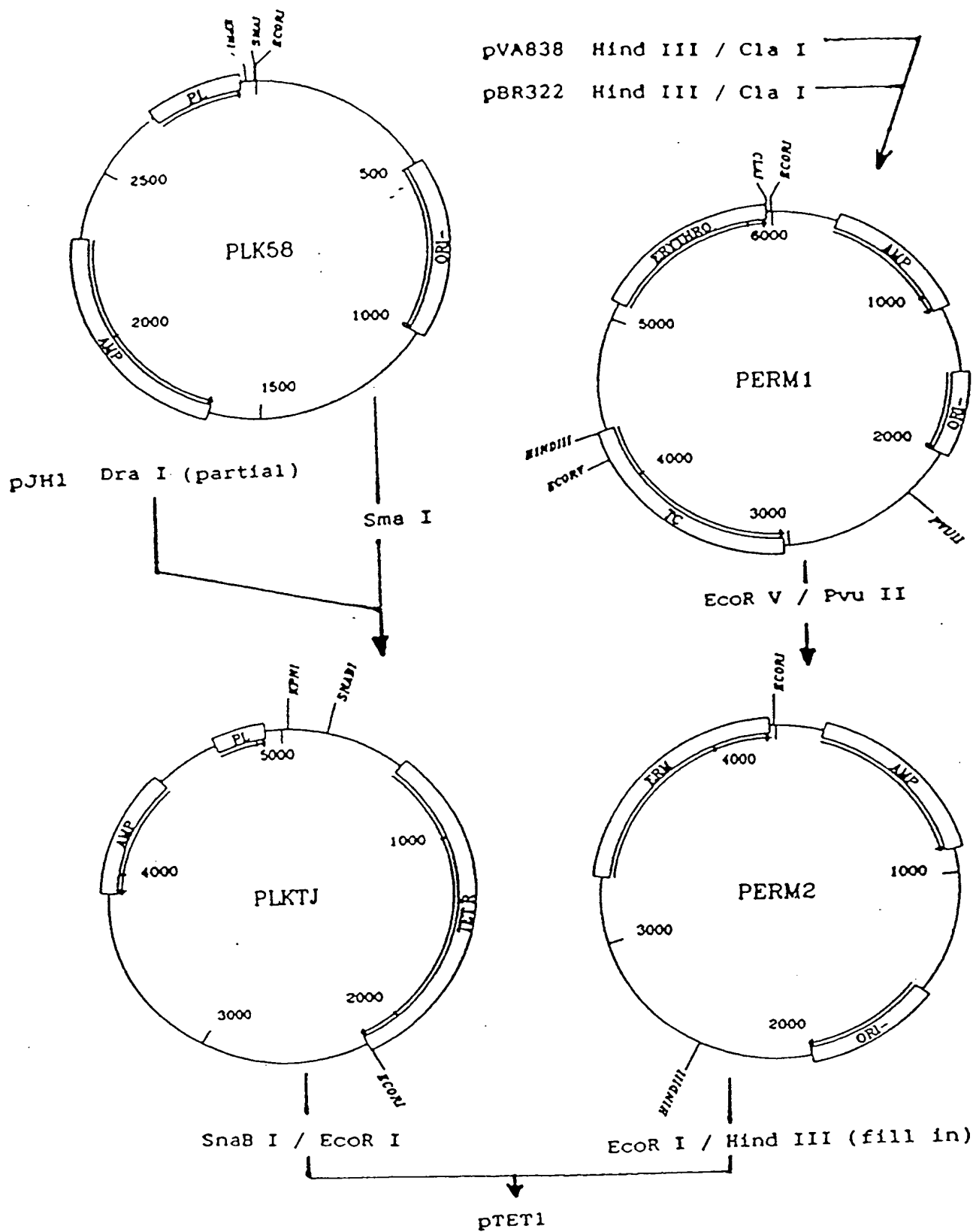


Fig. 10 continued

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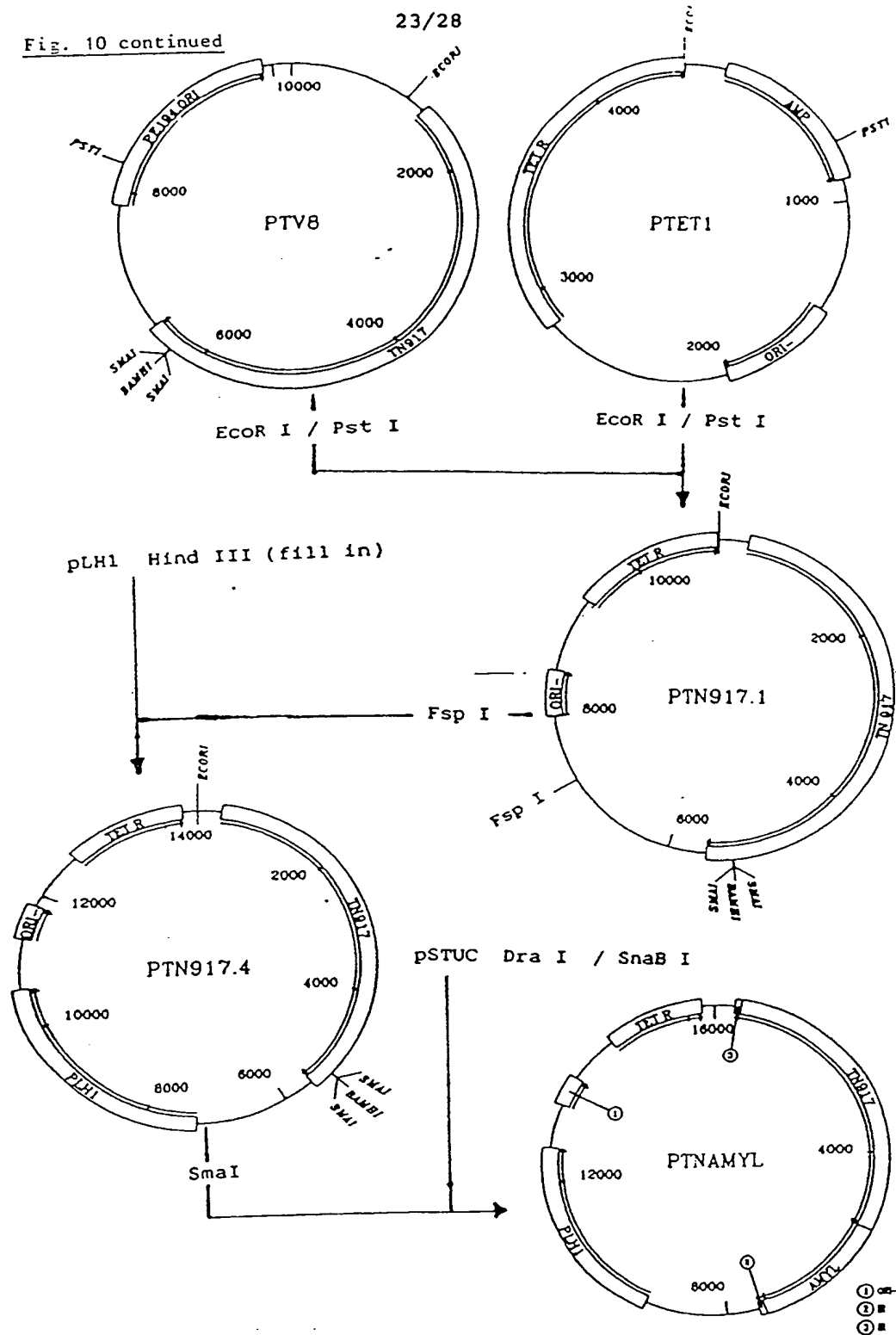


Fig. 11

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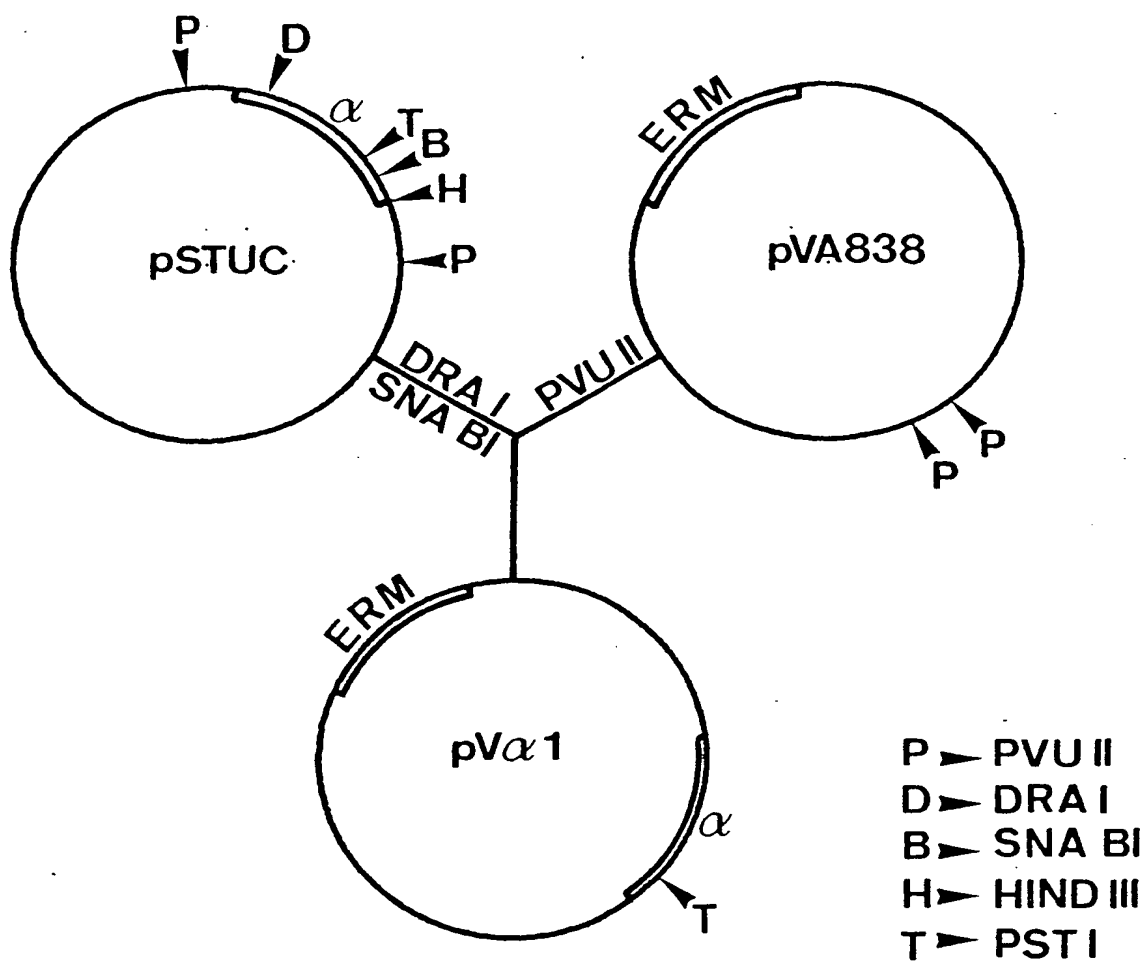
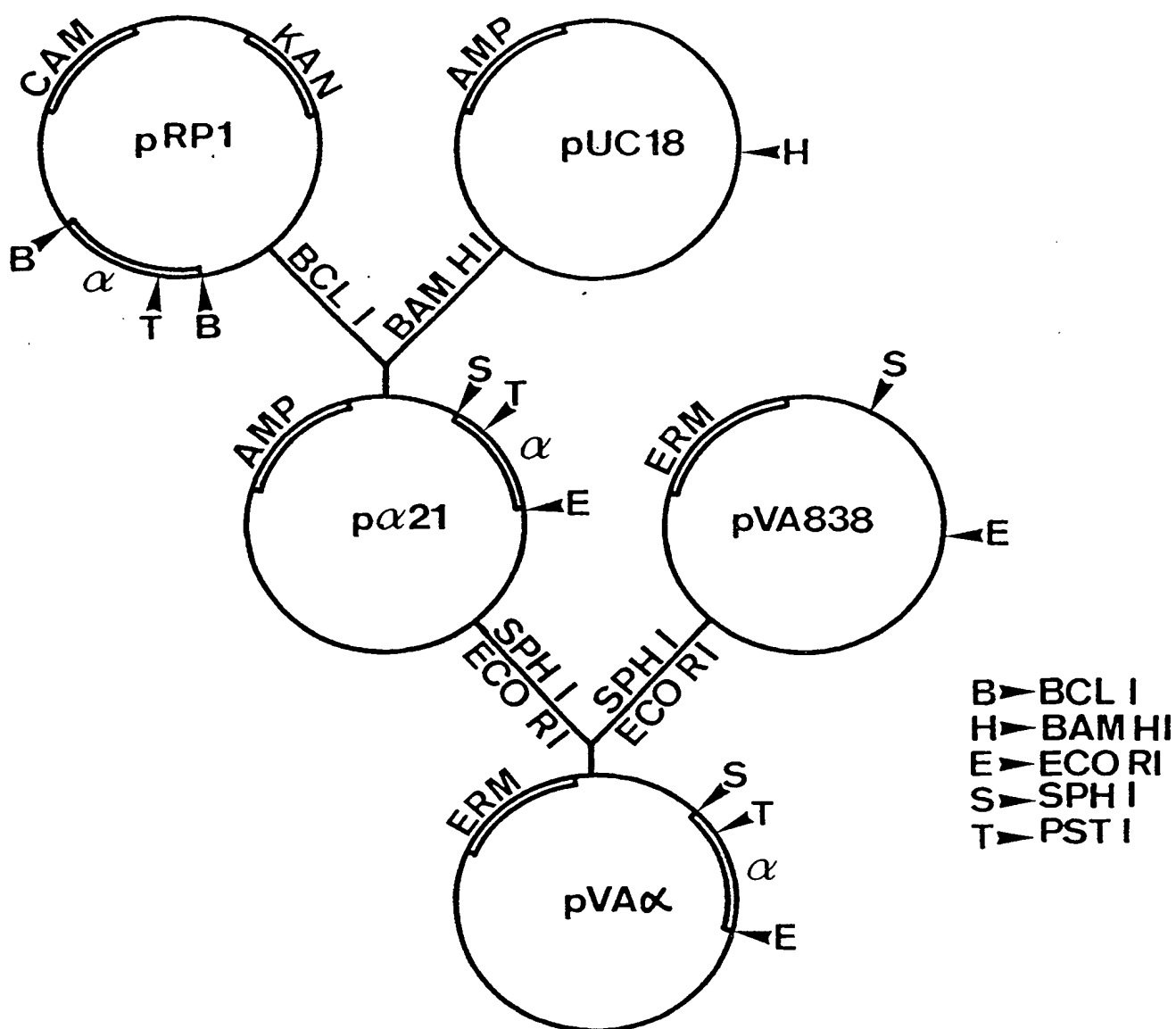


Fig. 12

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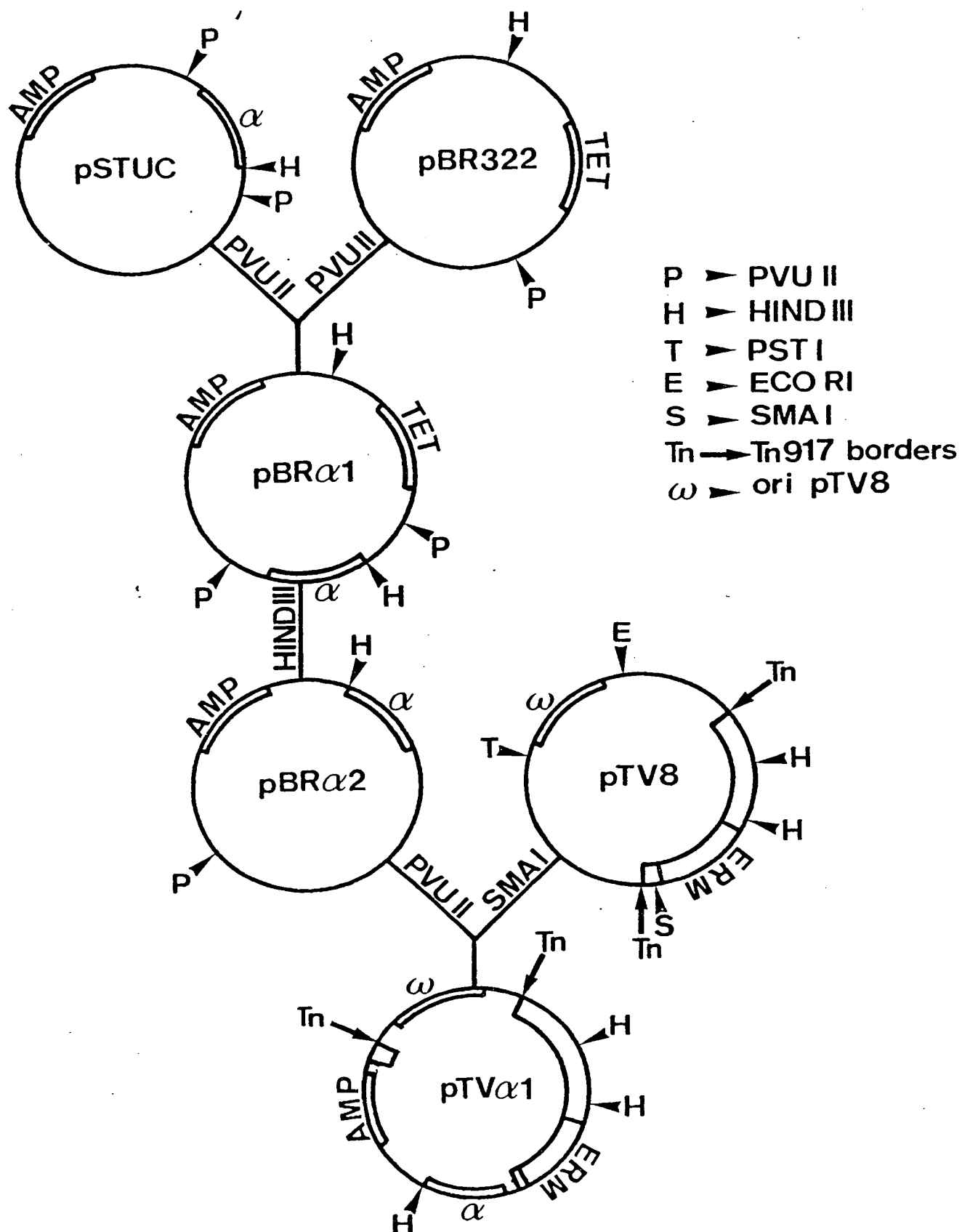


Fig. 14

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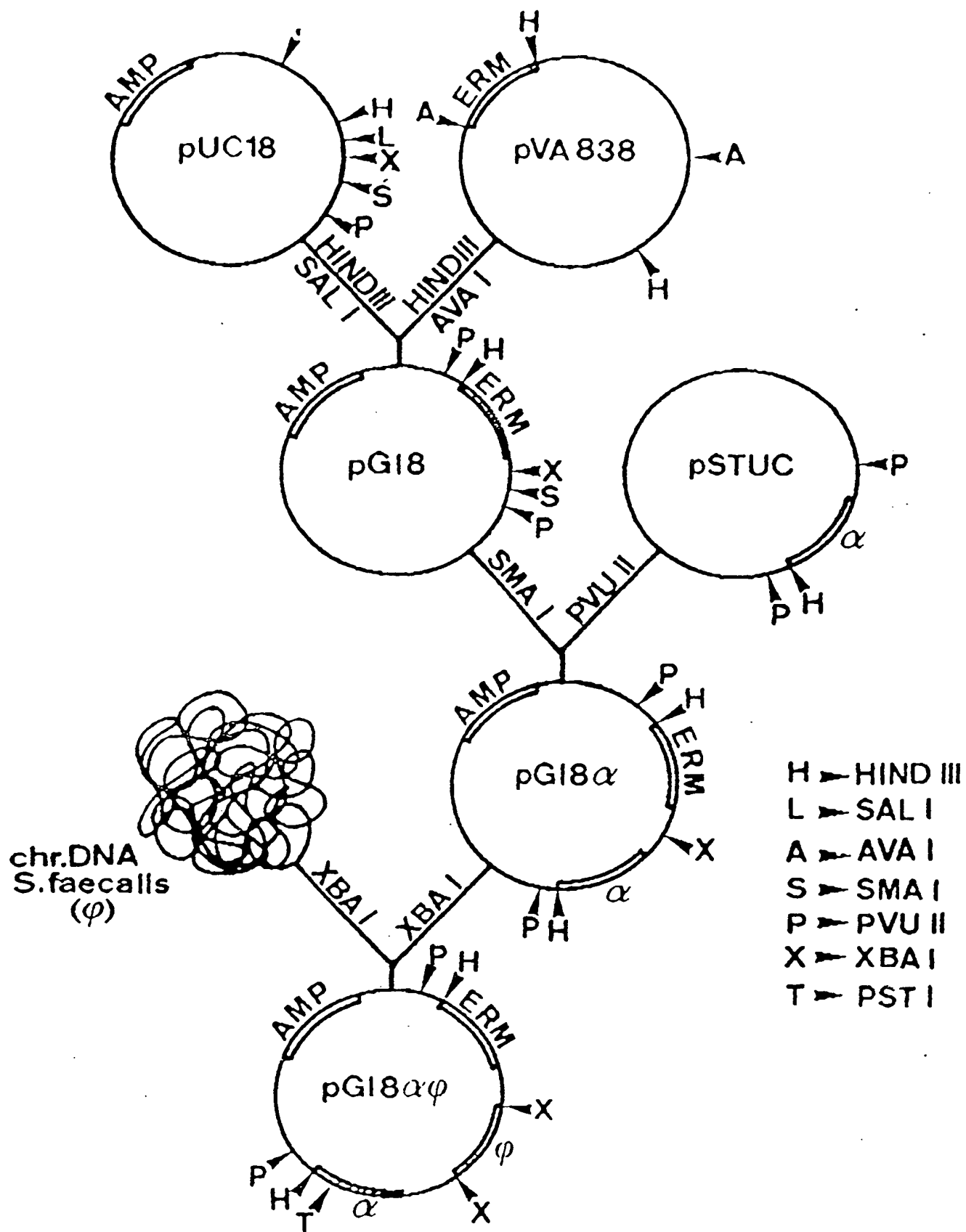
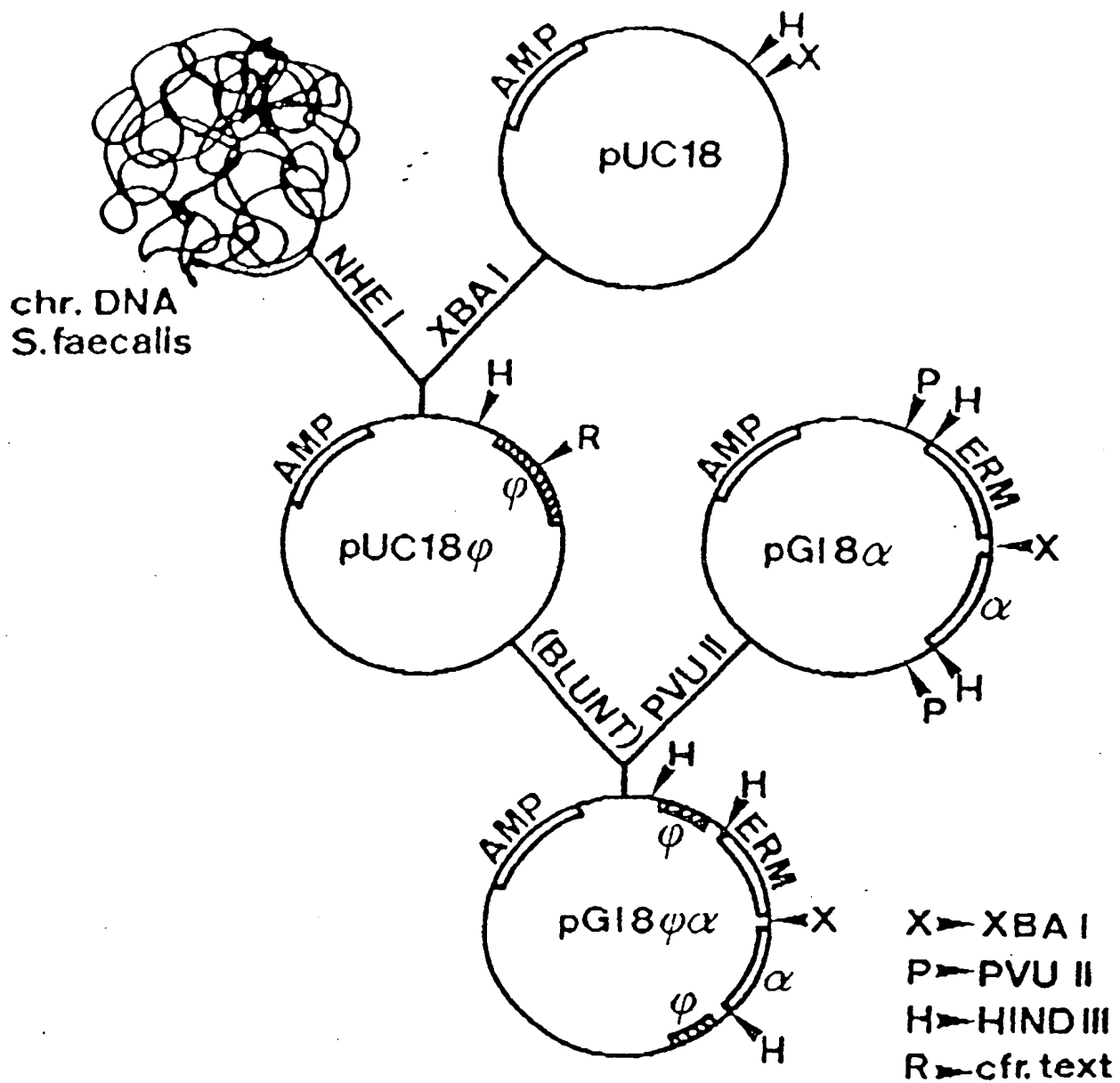


Fig. 15

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(19)



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Office européen des brevets

(11) Publication number:

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A3**

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A23L 1/03, A61K 35/74,
C12Q 1/40, C12N 9/28,
C12N 1/20, //(C12N1/20,
C12R1:01,1:46,1:225)**

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(96) Date of deferred publication of the search report:
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(54) Transformed lactic acid bacteria.

(57) An inoculum for silage and a probiotic which include lactic acid bacteria transformed with at least one exogenous gene or DNA fragment thereof coding for an enzyme which breaks down an oligosaccharide and/or a polysaccharide into a monosaccharide, disaccharide or other fermentable carbohydrate. Also provided are methods for transforming the lactic acid bacteria by electroporation and by the use of new plasmids, vectors and other DNA sequences. A new amylase is also provided.

EP 0 311 469 A3



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A, D	JOURNAL OF APPLIED BACTERIOLOGY SYMPOSIUM SUPPLEMENT, 1986, Oxford, GB D.R. SEALE: "Bacterial inoculants as silage additives", * Page 22S, lines 27-34 * --	1-4, 8, 11	C 12 N 15/00 A 23 K 3/02 A 23 L 1/03 A 61 K 35/74 C 12 Q 1/40 C 12 N 9/28 C 12 N 1/20// (C 12 N 1/20, C 12 R 1:01, 1:46, 1:225)
A	CHEMICAL ABSTRACTS, vol. 91, no. 25, December 17, 1979, page 516, abstract 209326j, Columbus, Ohio, US J.F. TOBEY et al.: "Factors affecting the production of amylase by Bacillus thuringiensis" & DEV. IND. MICROBIOL., 1976, (Publ. 1977), 18, 499-510 * Abstract * --	2	
A	FOOD TECHNOLOGY, vol. 40, no. 10, October 1986, pages 95-98, Chicago, Ill., US C.A. BATT: "Genetic engineering of Lactobacillus" * Page 96, column 1, line 32 - page 98, column 1, line 57 * --	1	TECHNICAL FIELDS SEARCHED (Int. Cl. 4) C 12 N A 23 K
A	ANNALS NEW YORK ACADEMY OF SCIENCE, vol. 469, 1986, pages 1-17, New York, US D.J. MCCONNELL et al.: "Genetic engineering of extra-cellular enzyme systems of Bacilli" * Page 1, line 20 - page 3, line 48; table 1 * -- ./.	2	
The present search report has been drawn up for all claims			
Place of search The Hague		Date of completion of the search 19-02-1990	Examiner VAN PUTTEN
<p>CATEGORY OF CITED DOCUMENTS</p> <p>X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document</p> <p>T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons A : member of the same patent family, corresponding document</p>			



CLAIMS INCURRING FEES

The present European patent application comprised at the time of filing more than ten claims.

- ☐ All claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for all claims.
- ☐ Only part of the claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims and for those claims for which claims fees have been paid.
- namely claims:
- ☐ No claims fees have been paid within the prescribed time limit. The present European search report has been drawn up for the first ten claims.

X LACK OF UNITY OF INVENTION

The Search Division considers that the present European patent application does not comply with the requirement of unity of invention and relates to several inventions or groups of inventions.

namely:

1. Claims 1-4, 8,11: An inoculum for silage. An ensilage method.
2. Claims 5,9: A proliotic. A method for establishing and maintaining optimal intestinal flora.
3. Claims 6,10: DNA which codes for an amylase. An amylase.
4. Claims 7,13,14: A plasmid DNA originating from *Lactobacillus* DNA comprising features derived from this plasmid.
5. Claim 12: A method for transforming lactic acid bacteria
6. Claim 15: A method for detecting a microorganism

- ☐ All further search fees have been paid within the fixed time limit. The present European search report has been drawn up for all claims.
- ☒ Only part of the further search fees have been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the inventions in respect of which search fees have been paid.
- namely claims: 1-5,8,9,11
- ☐ None of the further search fees has been paid within the fixed time limit. The present European search report has been drawn up for those parts of the European patent application which relate to the invention first mentioned in the claims.
- namely claims:



DOCUMENTS CONSIDERED TO BE RELEVANT			
Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int. Cl. 4)
A	EP-A-0 195 672 (A. MATSUSHIRO) * Examples 2,4,6-9 *	1	
	--		
T,D	DEVELOPMENTS IN SILAGE; Ed. J.M. WILKINSON & BASTARK; Chalcombe Publ. 1987, pages 47-62, D.R. SEALE: "Bacteria and enzymes as products to improve silage preservation" * Whole document *	1-4,8, 11	
	--		
E	NL-A-87 00 890 (TNO) * Page 1, line 9 - page 2, line 30; page 13, line 8 - page 14, line 12 *	1-5,8, 9,11	
	--		
T,D	FEMS MICROBIOLOGY REVIEWS, vol. 46, 1987, pages 343-356, Elsevier, A'dam, NL; C.F. FERNANDES et al.: "Therapeutic role of dietary lactobacilli and lactobacillic fermented dairy pro- ducts" * Whole article *	5,9	

The present search report has been drawn up for all claims			
Place of search		Date of completion of the search	Examiner
CATEGORY OF CITED DOCUMENTS			
X : particularly relevant if taken alone Y : particularly relevant if combined with another document of the same category A : technological background O : non-written disclosure P : intermediate document		T : theory or principle underlying the invention E : earlier patent document, but published on, or after the filing date D : document cited in the application L : document cited for other reasons & : member of the same patent family, corresponding document	